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Regulation of Serum Albumin Production by Insulin

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Abstract
Diabetes is accompanied by dysregulation of glucose, lipid, and protein metabolism. In recent years, much effort has been spent on understanding how insulin regulates glucose and lipid metabolism, while the effect of insulin on protein metabolism has received less attention. In diabetes, hepatic production of serum albumin decreases, and it has long been established that insulin positively controls albumin gene expression. Yet, the detailed pathway via which insulin exerts this effect has not been described. In this study, we used a genetic approach in mice to identify the mechanism by which insulin regulates albumin production, both transcriptionally and post-transcriptionally. Albumin expression was significantly decreased in livers with insulin signaling disrupted by ablation of insulin receptor or Akt. Concomitant deletion of Forkhead Box O1 (Foxo1) in these livers rescued the decreased albumin secretion. Furthermore, expressing a constitutively active Foxo1 in the liver is sufficient to suppress albumin expression. Mammalian Target of Rapamacin Complex 1 (mTORC1) activity had a minor contribution to serum albumin production. Hepatic autophagy also played a minor role and contributed to albumin production post-transcriptionally in the absence of insulin signaling. In addition, we show that constitutive activation of Foxo1 is correlated with decreased occupancy of CCAAT/Enhancer Binding Protein α (C/EBPα) at the albumin promoter. These results demonstrate that serum albumin production is regulated mainly on the transcription level, and insulin stimulates albumin expression by inhibiting Foxo1, which acts as a gene repressor of albumin by directly or indirectly interfering with C/EBPα binding to the albumin promoter.

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ABSTRACT

REGULATION OF SERUM ALBUMIN PRODUCTION BY INSULIN

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Diabetes is accompanied by dysregulation of glucose, lipid, and protein metabolism. In recent years, much effort has been spent on understanding how insulin regulates glucose and lipid metabolism, while the effect of insulin on protein metabolism has received less attention. In diabetes, hepatic production of serum albumin decreases, and it has long been established that insulin positively controls albumin gene expression. Yet, the detailed pathway via which insulin exerts this effect has not been described. In this study, we used a genetic approach in mice to identify the mechanism by which insulin regulates albumin production, both transcriptionally and post-transcriptionally. Albumin expression was significantly decreased in livers with insulin signaling disrupted by ablation of insulin receptor or Akt. Concomitant deletion of Forkhead Box O1 (*Foxo1*) in these livers rescued the decreased albumin secretion. Furthermore, expressing a constitutively active Foxo1 in the liver is sufficient to suppress albumin expression. Mammalian Target of Rapamacin Complex 1 (mTORC1) activity had a minor contribution to serum albumin production. Hepatic autophagy also played a minor role and contributed to albumin production post-transcriptionally

in the absence of insulin signaling. In addition, we show that constitutive activation of Foxo1 is correlated with decreased occupancy of CCAAT/Enhancer Binding Protein α (C/EBPα) at the albumin promoter. These results demonstrate that serum albumin production is regulated mainly on the transcription level, and insulin stimulates albumin expression by inhibiting Foxo1, which acts as a gene repressor of albumin by directly or indirectly interfering with C/EBPα binding to the albumin promoter.

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Chapter 1

General Introduction

Serum albumin: function, production, and regulation

Physiological function of serum albumin

Albumin is the most abundant plasma protein, accounting for ~60% of all proteins in circulation. Synthesized solely in the liver at a high rate (12-25 grams per day in a healthy adult), albumin has important physiological functions and is also implicated in various disease states (Fanali et al., 2012). Serum albumin is the main determinant of colloid osmotic pressure and modulates the fluid distribution between intravascular and extravascular compartments. With its binding capacity for a myriad of ligands, albumin serves as a key carrier for both endogenous biomolecules, including cholesterol, fatty acids, and metal ions, and exogenous compounds such as drugs. In addition, albumin exhibits anti-oxidant effects and also functions as a free-radical scavenger to maintain a balanced oxidative environment in plasma (Evans, 2002; Fanali et al., 2012; Nicholson, 2000; Peters, 1995).

In the clinical setting, serum albumin is frequently used as a cheap and reliable biomarker for various diseases. For instance, due to its elevated uptake into solid tumors and sites with high levels of inflammation, low albumin is proven to be a useful indicator for cancer and rheumatoid arthritis. In addition to its application in diagnosis, albumin is often administered to treat and manage various diseases including burns, hemorrhage, and liver disease (Evans, 2002; Fanali et al., 2012; Nicholson, 2000; Peters, 1995). Furthermore, albumin has the potential to function as an effective drug carrier (Kratz, 2008).

Regulation of albumin production

The colloid osmotic pressure of the interstitial fluid surrounding the hepatocytes is the most important regulator for albumin production. Indeed, intravenous infusion in rats with macromolecules, such as albumin, globulins, and dextran, cause a decrease in transcriptional activity of albumin (Pietrangelo et al., 1992). This observation demonstrates an effective feedback mechanism to maintain the homeostatic concentration of albumin in circulation.

The proper hormonal environment is also essential for optimal albumin synthesis. Insulin is required for adequate albumin synthesis and controls albumin production at the transcription level (discussed in more details below). Glucocorticoids are also an essential regulator for albumin transcription, as adrenalectomized rats exhibit decreased albumin expression (Nawa et al., 1986). Consistent with this result, albumin transcription in primary rat hepatocytes can be stimulated by dexamethasone treatment (Hutson et al., 1987; Kimball et al., 1995; Nawa et al., 1986). Growth hormone, when added to primary hepatocyte culture, also has a stimulatory effect on albumin expression (Johnson et al., 1991). Albumin synthesis and secretion are inhibited by glucagon (Dich and Gluud, 1976; Masumoto et al., 1988; Tavill et al., 1973; Uchida et al., 1991), although the inhibitory effect of glucagon on albumin production is likely post transcriptional: Nawa et al. found that while glucagon enhances albumin gene transcription induced by dexamethasone, glucagon alone has no effect on albumin expression (Nawa et al., 1986).

Finally, nutritional state plays a significant role in albumin production. Prolonged starvation decreases albumin production in liver (Kelman et al., 1972). This is possibly due to limited availability of amino acids under this condition, as it was observed that culturing primary rat hepatocytes in amino acids-deficient media leads to decreased albumin mRNA and secretion (Hutson et al., 1987; Kelman et al., 1972; Nawa et al., 1986). Among essential amino acids, leucine, isoleucine, and tryptophan have stimulatory effects on albumin production (Hutson et al., 1987). Amino acids also regulate albumin synthesis *in vivo*, as rats fed a low-protein diet exhibit reduced albumin expression and protein secretion (Nawa et al., 1986).

Regulation of albumin production by insulin

Over 30 years ago, Peavy and colleagues found that albumin production is significantly reduced in alloxan-induced Type 1 diabetic rats. Parallel with this change, abundance of albumin mRNA in the liver is also reduced. Administration of insulin in these animals can restore both albumin mRNA and secretion (Peavy et al., 1978). A similar observation was made in spontaneously diabetic rats, where insulin deficiency causes a decreases in albumin mRNA and synthesis, and this defect is eliminated with insulin treatment (Jefferson et al., 1983). These studies highlight the stimulatory effect of insulin on hepatic albumin production by regulating the quantity of albumin mRNA.

More biochemical studies have since demonstrated that insulin stimulates albumin production on the level of transcription. Since the apparent half-life of albumin mRNA is unaltered in diabetic rats, degradation of mRNA is unlikely to be the underlying mechanism. Furthermore, the size of albumin-synthesizing polysomes and the ribosomal half-transit time are comparable between diabetic and control rats, suggesting that the translation efficiency of the albumin message is not changed (Peavy et al., 1985). Using primary rat hepatocyte culture (Flaim et al., 1985), Lloyd et al. definitely showed that insulin stimulates albumin transcription, which directly correlates with albumin mRNA and secretion levels (Lloyd et al., 1987).

Consistent with observations made in animal models, Type 1 diabetic patients undergoing an insulin withdrawal exhibit significantly reduced albumin secretion, indicating that insulin positively regulates albumin production in human subjects as well (De Feo et al., 1991). Defects in serum albumin production might be specific to the type of diabetes, as the albumin synthetic rate is normal in Type 2 diabetic patients, and insulin can stimulate albumin production to the same extent in Type 2 diabetic patients as in control subjects (Tessari et al., 2006b). This observation suggests that the regulation of albumin production by insulin is intact in Type 2 diabetes, which could be explained by the model of selective insulin (See Chapter 2, “Discussion”, and Chapter 4, “Serum albumin production is intact in Type 2 diabetes”).

Significance

As described previously, serum albumin performs a myriad of important physiological functions, including controlling oncotic pressure and modulating drug metabolism. Under conditions of malnutrition, caused by diabetes, starvation, or protein deficiency, albumin expression and production are decreased. Hypoalbuminemia, often manifested as swelling, muscular symptoms, loss of appetite, ascites, and pleural effusions, would lead to dysregulation of albumin-mediated processes. Since treatment of hypoalbuminemia requires treating the underlying cause, it is therefore crucial to understand the regulatory mechanism of albumin production in order to devise the most effective treatment paradigm. This study will shed light on the molecular basis of how serum albumin production is regulated by insulin.

Insulin signaling and its physiological function

Overview of the physiological role of insulin

Insulin is a pivotal hormone that regulates metabolism and growth (Bedinger and Adams, 2015; Biddinger and Kahn, 2006; Saltiel and Kahn, 2001). Upon nutrient influx, insulin is secreted from pancreatic β-cells in response to the rising blood glucose level. In peripheral tissues such as fat and muscle, insulin stimulates nutrient uptake and storage, while inhibiting nutrient breakdown and release. Specifically, insulin promotes glucose uptake into cells by mediating the translocation of the glucose transporter, GLUT4, to the cell membrane. In adipocytes, insulin activates the lipogenic pathway to convert glucose to

triglycerides for long-term energy storage. Simultaneously, insulin inhibits lipolysis via inactivation of hormone-sensitive lipase (HSL), thereby preventing the release of free fatty acids and glycerol into circulation. In muscle, insulin stimulates the net production of glycogen from dietary glucose (Bedinger and Adams, 2015; Biddinger and Kahn, 2006; Saltiel and Kahn, 2001). Liver is another major site of insulin action. Hepatic glucose production is turned off by insulin as a result of its inhibition of gluconeogenesis and glycogenolysis. Moreover, insulin signaling switches the liver from fatty acids oxidation to a net production and secretion of lipids (Bedinger and Adams, 2015; Biddinger and Kahn, 2006). Overall, insulin mediates the whole-body postprandial response and maintains glucose and lipid homeostasis.

The insulin signaling pathway

The insulin signaling cascade is initiated by insulin binding to its receptor. The insulin receptor (IR) is a heterodimeric complex, and each dimer contains an α subunit and a β subunit. The β subunits of IR contain intrinsic tyrosine kinase activity that is repressed by the α subunits in the absence of insulin. Upon insulin binding to the α subunits, the β subunits become derepressed and transphosphorylate each other, resulting in full activation of IR. Downstream of IR, tyrosine residues on insulin receptor substrate (IRS) are phosphorylated and serve as docking sites for proteins containing Src homology 2 (SH2) domains. In the case of insulin signaling, the protein that docks on phosphorylated IRS is phosphatidylinositol 3-kinase (PI3K), which phosphorylates phosphoinositides at

the 3-position to produce PIP3. PIP3 binds various signaling molecules via their pleckstrin homology (PH) domains, thereby activating them or changing their cellular localization. Downstream of PI3K activation and PIP3 generation, two events occur that lead to the activation of Akt or protein kinase B (PKB): First, phosphoinositide-dependent kinase 1 (PDK1), the upstream activator of Akt, becomes activated as a result of PIP3 accumulation; Second, Akt is recruited to the plasma membrane in the vicinity of PDK1 to be phosphorylated at Thr308 and activated (Biddinger and Kahn, 2006; Saltiel and Kahn, 2001). The Rictor-containing mammalian target of rapamycin complex (mTORC2) also directly phosphorylates Akt at Ser473 for its full activation (Sarbassov et al., 2005). Although phosphorylation at Ser473 is not required for phosphorylation at Thr308, mTORC2-mediated phosphorylation seems to determine Akt specificity (Jacinto et al., 2006; Shiota et al., 2006).

Several pathways downstream of Akt mediate insulin's effects on metabolism. Akt phosphorylates and inactivates glycogen synthase kinase 3 (GSK3), relieving the repression on glycogen synthase and thus promoting glycogen synthesis (Cross et al., 1995). On another branch, Akt phosphorylates and inactivates the Tuberous Sclerosis 1/2 complex (TSC1/2), thereby releasing the inhibition of mammalian target of rapamycin complex 1 (mTORC1) (Inoki et al., 2002; Sengupta et al., 2010). Akt also activates mTORC1 by phosphorylating proline-rich Akt substrate of 40kDa (PRAS40), causing it to dissociate from mTORC1 and relieving its inhibitory constraint on mTORC1 activity (Wiza et al., 2012). In addition to Akt signaling, mTORC1 activity is

stimulated by amino acids (Sancak et al., 2010). AMP-activated protein kinase (AMPK), when activated by depleted cellular energy levels, phosphorylates the mTOR binding partner Raptor and leads to inactivation of mTORC1 (Gwinn et al., 2008). Downstream of insulin signaling, activation of mTORC1 stimulates protein synthesis as well as lipogenesis (Düvel et al., 2010; Laplante and Sabatini, 2010; Li et al., 2010; Thoreen et al., 2012; Wang and Proud, 2006). Finally, Akt phosphorylates the transcription factor forkhead box O1 (Foxo1), causing its translocation out of the nucleus (Biggs et al., 1999; Nakae et al., 1999; Rena, 2002; Rena et al., 1999). Foxo1 binds directly to the insulin response elements (IREs) in the promoters of key gluconeogenic enzymes to stimulate the expression of these genes under fasting conditions. Under postprandial conditions, when insulin is present, Foxo1 is located largely in the cytoplasm and thus becomes inactive as a transcription factor (Hall et al., 2000; Schmoll et al., 2000). Foxo1 can act as either a transcription activator or a repressor, although the detailed mechanism by which it represses gene expression is not yet fully understood (see below).

Impairment of insulin signaling and the development of Type 2 diabetes

Diabetes is a growing pandemic, affecting about 29 million people in the United States and creating a huge economic toll on the health care system. The disease is caused by failed production of (Type 1 diabetes) or dampened response to insulin (Type 2 diabetes). In Type 2 diabetes, insulin action is reduced, causing dysregulation of glucose and lipid metabolism. As a result,

Type 2 diabetic patients exhibit hyperglycemia and hyperlipidemia. To compensate for insulin resistance, insulin secretion from the pancreas is increased, resulting in hyperinsulinemia. Although both *in vivo* and *in vitro* studies have contributed to the understanding of the pathogenesis of insulin resistance and Type 2 diabetes, much of this complex metabolic disease remains to be understood (Bedinger and Adams, 2015; Biddinger and Kahn, 2006; Saltiel and Kahn, 2001).

Foxo1 as a transcriptional repressor

Overview

Genetic studies in C. *elegans* suggest that DAF-16, the Foxo ortholog, functions as a transcriptional activator downstream of the insulin/IGF-1 signaling (IIS) pathway by binding directly to the insulin response elements (IREs) in the promoter. However, DNA binding is not required for DAF-16 to repress gene expression for a different set of genes. Therefore, genes that are downregulated by DAF-16 (Class II genes) are more likely indirect targets (Murphy et al., 2003; Schuster et al., 2010; Tepper et al., 2013). Recently, Tepper et al. described an elusive transcriptional activator, PQM-1, that is mutually antagonistic with DAF-16 with regard to subcellular localization in response to IIS, providing a mechanism for the regulation of Class II genes (Tepper et al., 2013). To date, it is unclear whether a similar mechanism exists in mammals. Recent studies have suggested that Foxo1 potentially functions as a repressor indirectly by either

inducing the expression of a transcriptional repressor, or modulating the activity of another transcription factor.

Foxo1 induces expression of transcription repressors

Small heterodimer partner (Shp) interacts with and represses a range of nuclear receptors and transcription factors including liver X receptor alpha (LXRα) and hepatic nuclear factor 4α (Hnf-4α) to regulate cholesterol catabolism (Boulias et al., 2005; Huang et al., 2007; Park et al., 2011). It has been demonstrated that Foxo1 directly binds to the promoter of Shp and activates its expression (Shin et al., 2012; Wei et al., 2011). In addition, Shp also plays a role in liver metabolism consistent with it being a direct downstream target of Foxo1. Shp is induced in *ob*/*ob* and diet-induced obese livers. Shp-transgenic mice exhibit elevated hepatic triglyceride and bile acid, whereas Shp-null mice are protected from diet-induced obesity and hepatic steatosis. Furthermore, deletion of *Shp* improves insulin sensitivity and completely reverses hepatic steatosis in *ob*/*ob* mice (Boulias et al., 2005; Huang et al., 2007; Park et al., 2011). Another transcriptional repressor, inhibitor of DNA binding protein 3 (Id3), a basic helix-loop-helix (bHLH) protein that forms heterodimer and inhibits other bHLH proteins, is also identified as a Foxo1 target that is implicated in liver regeneration and development. (Shin et al., 2012). Interestingly, both Shp and Id3 might be involved in the regulation of albumin expression. Park et al. found that Shp physically interacts with C/EBPα, a transcriptional activator known to regulate albumin gene expression (see below) and represses its activity (Park et

al., 2007). In chick liver development, Id3 and albumin show reciprocal expressions both spatially and temporally (Nakayama et al., 2006).

Foxo1 modulates activity of other transcription factors

Ramaswamy et al. first demonstrated that Foxo1 can regulate gene expression independent of DNA-binding (Ramaswamy et al., 2002). Using a mutant Foxo1 that lacks DNA binding, the authors identified three classes of genes regulated by Foxo1 through transcriptional profiling. Class I genes are induced by Foxo1, and such induction requires DNA binding of Foxo1. Interestingly, a Foxo1 mutant lacking DNA-binding is able to induce and repress Class II and Class III genes, respectively. Moreover, chromatin immunoprecipitation using an antibody against Foxo1 shows that mutant Foxo1 is recruited to the promoter of Class II and Class III genes. These results suggest a mechanism where Foxo1 regulates gene expression by interacting with other transcription factors at the promoter. Indeed, it has been since discovered that Foxo1 directly interacts with a wide range of transcription factors to modulate their activity (Van der Vos and Coffer, 2008). For example, Foxo1 antagonizes the transcription activator activity of peroxisome proliferator-activated receptor γ (PPARγ), a critical regulator of adipocyte differentiation, by directly interacting with PPARγ and disrupting its DNA binding to the target genes (Dowell, 2003; Fan et al., 2009). Similarly, Deng et al. recently described a mechanism where Foxo1 directly interacts with Srebp-1c and interferes with its

binding to the *Srebp1c* promoter, thereby down regulating lipogenic genes (Deng et al., 2012).

Transcription factors known to regulate albumin gene expression

Overview

The promoter region of the albumin gene contains six distinct elements (Site A-F), recognized by several transcription factors (Lichtsteiner et al., 1987; Maire et al., 1989). Site B and Site D have the highest activating potential, whereas the rest of the sites have much less contribution to the promoter activity. Moreover, Site B and Site D interact with liver-enriched transcription factors known to regulate albumin gene expression, including hepatocyte nuclear factor 1α (Hnf-1α), CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, and D site binding protein (Dbp) (Lichtsteiner et al., 1987; Maire et al., 1989).

Hepatic nuclear factor 1α (Hnf-1α)

Predominantly expressed in liver and kidney, Hnf-1α is a transcription activator known to interact with many liver-specific genes, including albumin (Tronche et al., 1989), β-fibrinogen (Courtois et al., 1987), and α1-antitrypsin (Courtois et al., 1987; Monaci et al., 1988). Systemic inactivation of Hnf-1α leads to stunted growth and increased mortality. Hnf-1α is also involved in cholesterol and amino acid metabolism, as *Hnf1a*-deficient mice exhibits severe hypercholesterolemia and hyperphenylalanemia. Importantly, genetic ablation of

Hnf1a leads to decreased hepatic expression of albumin as well as lower serum albumin level (Lee et al., 1998; Pontoglio et al., 1996). These observations highlight an important regulatory role of Hnf-1α on albumin gene expression.

D site binding protein (Dbp)

Dbp, a member of the B-ZIP family, binds to the D site of the albumin promoter via its basic domain (Maire et al., 1989; Mueller et al., 1990). Interestingly, accumulation of Dbp mRNA and protein follow a "free-running" circadian pattern that is independent of feeding and drinking behaviors, where Dbp mRNA and protein levels are the highest in the evening and are the lowest in the morning (Wuarin and Schibler, 1990). Since *Dbp* knockout mice are still rhythmic, Dbp is most likely the output of circadian pathway rather than a regulator (Lopez-Molina, 1997). As a result of the circadian pattern of Dbp, albumin expression is the most efficient in the evening, as demonstrated by run-on experiments. Nevertheless, albumin expression does not fluctuate, probably due to the long half-life of its mRNA (Wuarin and Schibler, 1990).

CCAAT/enhancer binding protein α and β (C/EBP α and β)

C/EBP α and β are key regulators to control cell differentiation and proliferation (Nerlov, 2008). Highly expressed in liver, adipose, and lung, these transcription factors play a significant role in liver metabolism, adipogenesis, female fertility, and hermatopoiesis. Structurally, C/EBPs contain a conserved

DNA-binding and dimerization domain, and a C-terminal basic region-leucine zipper (bZIP) domain that mediates protein-protein interaction with other transcription factors (Nerlov, 2007), which provides an additional layer of regulation for C/EBPs function (see below). In addition, both C/EBPα and C/EBPβ exist in multiple isoforms as a result of alternative translation initiation. Work by Calkhoven et al. demonstrates that the relative ratio of C/EBP isoforms controls cell differentiation and cell fate (Calkhoven et al.).

C/EBPα plays a significant role in regulating whole-body metabolism. Congenital whole-body *Cebpa* knockout mice die shortly after birth due to defects in glycogen storage in the liver and subsequently hypoglycemia, suggesting that C/EBPα is required for mediating energy homeostasis in neonates (Wang et al., 1995). In liver, in addition to being a potent *trans*-activator for the albumin gene (Friedman et al., 1989), C/EBPα is also important in other liver functions, including bilirubin clearance and ammonia detoxification (Inoue et al., 2004; Y H Lee, 1997). Furthermore, C/EBPα is also a key mediator of glucose and lipid metabolism in liver, regulating the expression of gluconeogenic and lipogenic enzymes to mediate glycogen synthesis, hepatic glucose production, and lipid synthesis in the liver (Inoue et al., 2004; Matsusue et al., 2004; Qiao, 2006; Y H Lee, 1997).

Often acting together with C/EBPα as a heterodimer, C/EBPβ plays similar roles in mediating glucose and lipid metabolism in liver (Croniger et al., 2000; 1997; Liu et al., 1999; Millward et al., 2007; Rahman et al., 2007; Schroeder-Gloeckler et al., 2007). Importantly, C/EBPβ exists in two isoforms: a 35kDa liver

activating protein (LAP) and a 20kDa liver inhibitory protein (LIP). LAP and LIP are translated from the same mRNA transcript as a result of leaky ribosome scanning. LIP, lacking the transactivation domain, exhibits higher binding affinity for DNA and functions as a dominant inhibitor of C/EBP family members (Descombes and Schibler, 1991). Therefore, the transcriptional activity of a C/EBPβ target genes is dependent on the LAP/LIP ratio (Nerlov, 2008; Van der Vos and Coffer, 2008). It has been previously shown that LAP/LIP ratio is altered during terminal liver differentiation, ER stress, and inflammation (Hu et al., 2004; Li et al., 2008; Luedde et al., 2004). However, the regulatory mechanism controlling the relative synthesis and degradation of LAP and LIP remains unclear.

Autophagy and its regulatory role in metabolism

Overview

Autophagy, which translates to “eating oneself”, is an evolutionarily conserved quality control process that degrades and recycles damaged cellular components and organelles, and the broken-down constituents are in turn used for either biosynthesis or energy generation (Kim and Lee, 2014; Rabinowitz and White, 2010; Yamada and Singh, 2012). There are three kinds of autophagy: Macroautophagy (referred to as “autophagy” hereafter), characterized by the formation of autophagosomes, is responsible for the turnover of organelles and proteins. Microautophagy, on the other hand, involves the formation of single-

membraned vesicles, which then pinch off within the lysosomal lumen for the degradation of the enclosed content (Sahu et al., 2011). Finally, chaperone-mediated autophagy is a degradation process selective for soluble cytosolic proteins with the KFERQ signature (Arias and Cuervo, 2011). Under basal conditions, constitutive autophagy plays an important housekeeping role to maintain cellular functions and energy balance. In the event of metabolic stress, such as starvation, the process of self-cannibalization is a key source of nutrients to meet the energy demand. Recently, numerous studies have demonstrated that autophagy is an essential mediator of energy homeostasis, and dysregulation of autophagy has direct implications in various diseases including metabolic disorders, neurodegenerative diseases, and cancer (Kim and Lee, 2014; Rabinowitz and White, 2010; Yamada and Singh, 2012).

Autophagy mediates whole-body metabolism

Dispensable during embryonic development, autophagy is induced at birth in various tissues, including heart, lung, and diaphragm, and remains high for 12 hours in neonates before returning to basal levels (Kuma et al., 2004). Autophagy-deficient mice exhibit significantly reduced level of amino acids and die within 1 day of birth (Komatsu, 2005; Kuma et al., 2004; Sou et al., 2008). Somewhat surprisingly, blood glucose and lipid levels of autophagy-deficient mice are comparable with wildtype littermates, suggesting that in neonates, the major role of autophagy is to provide amino acids from breaking down in-house proteins (Kuma et al., 2004). These studies have demonstrated that neonates

utilize autophagy to maintain energy balance at birth, when nutrient supply is suddenly disrupted.

Autophagy has also been implicated in aging. Well-established regimens that extend life span, including caloric restriction, TOR inhibition, and genetic manipulation of the insulin signaling cascade, all stimulate the autophagy pathway. Furthermore, in many of these cases, the longevity-promoting effects are dependent on autophagy (Madeo et al., 2010; Rubinsztein et al., 2011). Recently, Pyo et al. demonstrated that ubiquitous overexpression of Atg5 in mice extends lifespan significantly (17.2%), and the animals exhibit a list of anti-aging phenotypes such as reduced adiposity, enhanced insulin sensitivity, and improved motor function (Pyo et al., 2013). These observations highlight the cytoprotective role of autophagy and further emphasize the important role of autophagy in maintaining metabolic homeostasis. The metabolic role of autophagy in adipose tissue and liver is summarized below.

Autophagy in adipose tissue

Autophagy in adipose tissue has a critical role in adipocyte differentiation and adipogenesis. Mice with fat-specific disruption of autophagy are lean and exhibit significantly reduced fat mass compared to wild type controls. Morphological studies reveal that autophagy-deficient adipocytes are smaller and have multilocular lipid droplets, larger cytosol, and increased number of mitochondria. Consistent with increased mitochondrial content, β-oxidation of fatty acids is increased in these animals. Furthermore, white adipose tissue with disrupted autophagy exhibit features of brown fat, including increased levels of

UCP1. Due to altered adipose physiology, fat-specific *Atg7* knockout mice have enhanced insulin sensitivity and are protected from high-fat-diet-induced obesity (Singh et al., 2009a; Zhang et al., 2009).

Autophagy in adipose tissue is also associated with obesity, insulin resistance, and the development of Type 2 diabetes. In both mice and human, autophagy in adipose tissue is elevated in obese subjects compared to lean controls. In addition, when compared to insulin-sensitive subjects, insulin-resistant individuals exhibit higher levels of autophagic activity in the adipose tissue (Jansen et al., 2012; Kovsan et al., 2010). Consistent with this observation, patients with Type 2 diabetes show strongly upregulated autophagy in their adipocytes (Anita Öst, 2010). One proposed mechanism by which autophagy contributes to the development of insulin resistance in adipocytes is mediating the degradation of insulin receptor, downstream of the elevated level of ER stress (Zhou et al., 2009).

Autophagy in liver

In liver, autophagy mediates energy metabolism in response to hormonal signals and nutrient availability (Yin et al., 2008). Mice with autophagy disrupted specifically in the liver exhibit severe hepatomegaly, increased hepatic protein content, and accumulation of deformed peroxisomes, mitochondria, and ER, suggesting that liver autophagy plays a significant role in the regulation of liver mass and the removal of dysfunctional organelles (Komatsu, 2005; Yin et al.,

2008). Hepatic autophagy also contributes to the metabolic processes in liver. Singh and colleagues discovered that autophagy negatively regulates the intracellular lipid stores in liver, a process they referred to as “macrolipophagy”. Consistent with this finding, they also observed that disruption of hepatic autophagy cause increased hepatic triglyceride content and total lipid droplets in hepatocytes (Singh et al., 2009a). In addition to lipid metabolism, autophagy also contributes to glucose production by supplying amino acids as gluconeogenic precursors during starvation (Ezaki et al., 2011).

In addition to having a metabolic role, autophagy also plays a significant regulatory role in mediating liver metabolism. Opposite of what is observed in the adipose tissue, hepatic autophagy is suppressed during obesity (Liu et al., 2009; Singh et al., 2009a; Yang et al., 2010). This indicates that the metabolic function of autophagy is tissue-specific. Recently, Yang et al. showed that suppressing autophagy impairs insulin signaling both *in vitro* and *in vivo*, as measured by phosphorylation of IR and Akt upon insulin stimulation. On the contrary, inducing hepatic autophagy in leptin-deficient *ob/ob* mice increases insulin signaling, reduces steatosis, and improves systemic glucose homeostasis (Yang et al., 2010). Thus, there might be value in targeting hepatic autophagy as a therapeutic intervention against obesity and insulin resistance.

Chapter 2

Insulin stimulates albumin gene expression by inhibiting Foxo1

Introduction

Synthesized solely in the liver, serum albumin is the most abundant circulating protein, accounting for ~60% of total serum proteins. In addition to being the major determinant of oncotic pressure, albumin also functions as the carrier for many endogenous and exogenous compounds, including free fatty acids, ions, and drugs. Clinically, albumin is a crucial biomarker used to assess liver function (Fanali et al., 2012). Multiple factors, including nutritional states, oncotic pressure, and hormonal factors, regulate albumin production (Kimball et al., 1995; Pietrangelo et al., 1992; Sakuma et al., 1987). In Type 1 diabetes, the concentration of albumin in blood is decreased, and administration of insulin is required to prevent hypoalbuminemia (De Feo et al., 1991; Jefferson et al., 1983). Early biochemical studies have shown that insulin stimulates albumin production in the liver by activating gene transcription (De Feo et al., 1991; Flaim et al., 1985; Hutson et al., 1987; Jefferson et al., 1983; Kimball et al., 1995; Lloyd et al., 1987; Peavy et al., 1978; 1985). Yet, the detailed pathway by which insulin exerts this effect has not been described.

In liver, insulin promotes protein production and lipid synthesis, while turning off gluconeogenesis and glycogenolysis (Brown and Goldstein, 2008; Cross et al., 1995; Leavens and Birnbaum, 2011; Saltiel and Kahn, 2001). The insulin signaling pathway has been well characterized: insulin binds to the insulin receptor (IR), which leads to phosphorylation of the insulin receptor substrate (IRS). This then initiates a cascade of signaling events that results in the phosphorylation and activation of Akt protein kinases (Saltiel and Kahn, 2001).

Several pathways downstream of Akt mediate insulin's effects on metabolism. Akt phosphorylates and inactivates the Tuberous Sclerosis 1/2 (TSC1/TSC2) complex, thereby releasing the inhibition of mammalian target of rapamycin complex 1 (mTORC1) (Inoki et al., 2002; Sengupta et al., 2010). Activation of mTORC1 stimulates protein synthesis as well as lipogenesis (Düvel et al., 2010; Laplante and Sabatini, 2010; Li et al., 2010; Thoreen et al., 2012; Wang and Proud, 2006). Akt also phosphorylates the transcription factor forkhead box O1 (Foxo1), causing its translocation out of the nucleus (Biggs et al., 1999; Nakae et al., 1999; Rena, 2002; Rena et al., 1999). Foxo1 binds directly to the insulin response elements (IREs) in the promoters of key gluconeogenic enzymes to stimulate the expression of these genes under fasting conditions. Under postprandial conditions when insulin is present, Foxo1 is located largely in the cytoplasm and thus becomes inactive as a transcription factor (Hall et al., 2000; Schmoll et al., 2000).

Autophagy, literally translated as "self-eating", is a mechanism where cellular constituents are engulfed in compartments called autophagosomes and delivered to the lysosome for degradation. Since its first description by de Duve and Wattiaux in 1966, autophagy has been extensively characterized. Autophagy has been implicated in human pathology, including neurodegenerative disease, cancer, and aging (Rabinowitz and White, 2010; Rubinsztein et al., 2011; Yamada and Singh, 2012). Moreover, autophagy is emerging as a critical component in the regulation of metabolism, as recent studies demonstrate its role in lipid and glucose metabolism (He et al., 2012; Liu

et al., 2009; Singh et al., 2009b; Yang et al., 2010). Furthermore, autophagy has been linked with pancreatic beta cell function and insulin action (Ebato et al., 2008; Jung et al., 2008). These observations suggest that autophagy, in addition to its cytoprotective role, is also an important metabolic modulator**.**

In the present study, we used a genetic approach to address the longstanding question of the mechanism by which insulin stimulates albumin transcription. We found that insulin acts directly on the liver through the IR/PI3K/Akt pathway to inhibit Foxo1, which functions as a repressor of albumin expression. Interestingly, elevated hepatic autophagy also contributes to serum albumin production, possibly by supplying amino acid, generating energy, and/or augmenting protein secretion.

Results

Albumin gene expression is reduced in Type 1, but not Type 2, diabetic livers

First, we assessed the effect of diabetes on albumin expression in mice. Steptozotocin (STZ), a compound that induces β-cell death, is frequently used to induce Type 1 diabetes in animal models. Mice injected with STZ developed severe hyperglycemia (Figure 2.1A) and lost a significant amount of body weight (Figure 2.1B). In addition, albumin gene expression in liver was significantly decreased compared to control animals (Figure 2.1C). Consistent with early studies in rats, this result suggests that insulin positively regulates hepatic albumin production on the level of transcription. We did not observe a difference

in serum albumin level between STZ-treated and control animals (not shown), possibly because the half-life of albumin protein is longer than the duration of the experiment (21 days and 11 days, respectively).

Next, we used the leptin-deficient (*ob*/*ob*) mouse model to address whether albumin production is also reduced in Type 2 diabetes. Interestingly, despite of having severe insulin resistance, *ob*/*ob* animals exhibited slightly elevated level of total serum protein and albumin gene expression compared to control (Figure 2.2 A and B). These results suggest that albumin transcription and production are slightly increased in Type 2 diabetic livers.

Insulin signals directly on the liver to stimulate albumin gene expression

To assess whether the regulation of insulin on albumin production is a cell-autonomous effect, we deleted insulin receptor (*Ir*) specifically in the liver (IRKO, Figure 2.3A). Serum albumin level was significantly reduced in IRKO animals compared to controls (GFP, Figure 2.3B). Consistent with the reduced circulating albumin protein level, albumin gene expression in IRKO livers was significantly decreased compared to controls (Figure 2.3C). These results suggest that insulin controls albumin production by signaling directly on the liver.

It is also worthwhile to note that a short-term (overnight) fast did not affect the steady-state serum albumin level or albumin expression level in either IRKO or control livers, as there was no significant difference between the overnight-fasted state and the fasted-refed state (Figure 2.3B and C). Rather than

measuring the newly synthesized species, here, total albumin protein and mRNA levels were measured. Since the half-life of these species exceeds the duration of overnight fasting, the stimulatory effect of insulin on albumin expression and protein level could not be observed.

Akt is required for proper albumin gene expression and production

Akt is an essential downstream signaling molecule that mediates various metabolic effects of insulin. We then investigated whether Akt is required to regulate albumin production downstream of IR. To this end, we deleted the only isoforms of *Akt* expressed in liver*, Akt1* and *Akt2*, specifically in the liver (AktDKO, Figure 2.4A). AktDKO mice exhibited a 50% reduction in serum albumin compared to control (GFP, Figure 2.4B). This severe hypoalbuminemia was correlated with a dramatic decrease in albumin gene expression (Figure 2.4C).

To address whether this albumin production defect is cell-autonomous, we isolated primary hepatocytes from GFP and AktDKO mice and measured albumin secretion *in vitro*. AktDKO hepatocytes secreted significantly less albumin compared to control (Figure 2.4D). Consistent with the reduced albumin secretion, albumin gene expression in AktDKO hepatocytes was significantly decreased (Figure 2.4E). Taken together, these results suggest that insulin signals through the Akt pathway to control albumin transcription and secretion.

Inhibition of Foxo1 is required for proper albumin expression and production

Foxo1, the transcription factor downstream of Akt, becomes constitutively active when insulin signaling is disrupted. We asked whether inhibition of Foxo1 as a result of insulin signaling is required for maintaining albumin transcription. Interestingly, additional deletion of *Foxo1* in IRKO livers (FoxoDKO, Figure 2.5A) fully restored the reduced circulating albumin level observed in IRKO mice (Figure 2.5B). In addition, albumin expression in FoxoDKO livers was completely restored to control levels (Figure 2.5C).

Similarly, additional deletion of *Foxo1* in AktDKO livers (FoxoTKO, Figure 2.6A) almost completely restored serum albumin level (Figure 2.6B), and completely restored the reduced albumin expression observed in AktDKO livers (Figure 2.6C). This effect was cell-autonomous: primary hepatocytes isolated from FoxoTKO livers exhibited comparable albumin secretion (Figure 2.6D) and albumin gene expression (Figure 2.6E) as control. These results suggest that insulin controls albumin transcription and secretion via, at least in part, the inhibition of Foxo1.

We then investigated whether inhibiting Foxo1 is sufficient to correct the reduced albumin expression in Type 1 diabetic livers. To this end, we used STZ to induce diabetes in either control (GFP) or liver-specific *Foxo1* knockout mice (FoxoKO, Figure 2.7A). Both genotypes developed severe hyperglycemia post STZ injection (Figure 2.7B). Importantly, STZ treatment in GFP mice caused a 50% reduction in albumin gene expression in liver, and this reduction was completely absent in FoxoKO mice (Figure 2.7C). In addition, we found that

transgenic mice expressing a constitutively active Foxo1 (CA-Foxo1) had significantly reduced hepatic albumin expression compared to wildtype controls (WT, Figure 2.8). These results suggest that constitutive activation of hepatic Foxo1 is sufficient to repress albumin expression and contributes to decreased albumin production in Typ1 diabetes.

Whether mTORC1 is required for proper albumin production is unclear

In addition to inhibiting Foxo1, Akt also phosphorylates and inhibits TSC1 to activate mTORC1, a major regulator for protein translation by activating S6 kinase. To investigate whether mTORC1 signaling also contributes to albumin production, we first assessed the effect of restoring the reduced hepatic mTORC1 activity in AktDKO mice. To this end, we concomitantly deleted *Tsc1* to generate liver-specific *Tsc1*/*Akt1*/*Akt2* triple-knockout mice (TSC1TKO, Figure 2.9A). As indicated by the constitutively elevated levels of ribosomal protein S6 phosphorylation, mTORC1 in these livers was constitutively active in the absence of hepatic Akt activity, regardless of the nutritional state (Figure 2.9A). While TSC1TKO animals exhibited significantly reduced serum protein level when compared to GFP controls, the defect was much milder than AktDKO animals (~25% vs. 50% reduction, Figure 2.9B). Furthermore, concomitant deletion of *Tsc1* showed no improvement on the reduced albumin gene expression observed in Akt-null livers (Figure 2.9C). These results suggest that mTORC1 signaling might contribute to hepatic albumin production post-transcriptionally.

However, we obtained a contradictory result when we directly examined the role of mTORC1 signaling on albumin production by deleting *Raptor*, an essential component of mTORC1, specifically in the liver (RaptorKO, Figure 2.10A). The absence of mTORC1 activity was confirmed by the complete lack of phosphorylated S6. In addition, as a result of decreased negative feedback mediated by S6 kinase, Akt was hyper-phosphorylated in RaptorKO livers (Figure 2.10A). Both total serum protein concentration and albumin gene expression level of RaptorKO mice were comparable to GFP controls (Figure 2.10B and C). These results suggest that mTORC1 activity is not required to maintain hepatic albumin production.

Elevated hepatic autophagy contributes to albumin production

Downstream of insulin/Akt signaling, mTORC1 inhibits autophagy, a process important for cellular renewal and waste disposal. Recent studies have shown that autophagy also plays a significant role in metabolic regulation and insulin action. Since mice lacking Akt in the liver exhibited impaired mTORC1 activity, hepatic autophagy in these animals was elevated, as indicated by the reduced level of p62, a protein degraded by autophagy (Figure 2.11). We then asked whether elevated autophagy contributes to the reduced albumin production in AktDKO animals.

First, we assessed the role of hepatic autophagy on albumin production in wildtype animals. To this end, we deleted *Atg5*, an essential component of the

autophagy pathway, specifically in the liver. Liver-specific Atg5 knockout (Atg5KO) animals exhibited normal blood glucose level and body weight (Figure 2.12A and B). Consistent with previous observations in models with disrupted hepatic autophagy, Atg5KO mice exhibited elevated liver weight compared to GFP controls (Figure 2.12C and D). Interestingly, Atg5KO mice showed a small decrease in total serum protein concentration in the overnight-fasted state but not in the fasted-refed state (Figure 2.12E). Since autophagy is elevated during fasting, this result suggests that active hepatic autophagy might contribute to albumin production. Furthermore, albumin gene expression in Atg5KO mice was normal (Figure 2.12F), suggesting that the role of autophagy on albumin production is post-transcriptional.

Next, we assessed the effect of elevated hepatic autophagy in AktDKO mice on albumin production. To this end, we concomitantly deleted *Atg5* and generated liver-specific *Atg5*/*Akt1*/*Akt2* triple-knockout mice (Atg5TKO, Figure 2.13A). Interestingly, Atg5TKO mice developed severe edema, accumulating a significant amount of fluid in the interstitial space (Figure 2.13B), indicating severe dysregulation of oncotic pressure, most likely due to defects in albumin production. Atg5TKO mice exhibited normal fasting glucose level and significantly increased body weight (Figure 2.13C and D). The body weight difference was mostly due to fluid accumulation (Figure 2.13E). Interestingly, the liver weight of Atg5TKO mice was comparable to GFP controls (Figure 2.13F). Since AktDKO livers were smaller, this result is consistent with previous

observation that disrupting hepatic autophagy leads to an increase in liver weight.

Disrupting hepatic autophagy in AktDKO livers exacerbated the reduced serum protein level (Figure 2.14A), consistent with the severe edema phenotype observed in Atg5TKO animals. Surprisingly, concomitant deletion of *Atg5* not only completely restored the reduced albumin gene expression observed in AktDKO livers, albumin mRNA levels in these livers were even slightly increased compared to GFP controls (Figure 2.14B).

Discussion

Early biochemical studies in rats have shown that insulin stimulates albumin production in liver by activating gene transcription (De Feo et al., 1991; Flaim et al., 1985; Hutson et al., 1987; Jefferson et al., 1983; Kimball et al., 1995; Lloyd et al., 1987; Peavy et al., 1978; 1985). Similarly, mice with STZ-induced Type 1 diabetes exhibited significantly reduced albumin gene expression compared to control (Figure 2.1C). Yet, the detailed pathway via which insulin regulates albumin gene expression has not been described. Here, we used a genetic approach to systematically elucidate this pathway.

Deletion of the insulin receptor specifically in the liver led to a decrease in both albumin gene expression and circulating albumin levels (Figure 2.3A and B), suggesting that insulin signals directly on the liver to control albumin production. This is consistent with previous observations described in Michael et al., where

the liver-specific congenital *Ir*-knockout mice exhibit a 50% reduction in serum albumin compared to wildtype control (Michael et al., 2000). The defect in our IRKO mice was much milder (~25% reduction, Figure 2.3B), possibly because our knockout model was acute and the animals were much younger (3 months old vs. 6 months old).

Disruption of both isoforms of hepatic *Akt* specifically in the liver also caused a significant reduction in both albumin gene expression and circulating albumin levels (Figure 2.4A and B), suggesting that Akt is required downstream of IR to mediate insulin's effect on albumin production. Importantly, when compared to the IRKO, AktDKO mice exhibited a larger defect, suggesting that basal hepatic Akt activity in IRKO mice maintained some albumin gene expression and protein production. Furthermore, Akt regulates albumin production cell-autonomously, as hepatocytes isolated from AktDKO animals exhibited decreased albumin gene expression and secreted less albumin protein *in vitro* compared to control (Figure 2.4D and E).

To interrogate how albumin production is regulated downstream of Akt, we first examined the role of Foxo1, a transcription factor phosphorylated and inhibited by Akt. Foxo1 is an important target whose inhibition mediates many of the actions of insulin. Liver-specific *Foxo1* knockout mice phenocopy the effect of insulin in having impaired glucose production (Matsumoto et al., 2007). In addition, inhibition of hepatic Foxo1 activity protects against high-fat diet induced hepatic insulin resistance (Kim et al., 2009; Xiong et al., 2013). On the other hand, transgenic mice with liver-specific expression of constitutively active Foxo1

exhibit fasting hyperglycemia, reduced *de novo* lipogenesis and hepatic insulin resistance (Zhang et al., 2006). These studies highlight that Foxo1 plays a significant role in regulating glucose and lipid metabolism downstream of insulin in liver. Here, we found that active Foxo1 also represses albumin expression. In models where Foxo1 was constitutively active (IRKO, AktDKO, STZ-induced diabetes), albumin gene expression was decreased and genetic ablation of *Foxo1* in these models completely restored the decreased albumin expression (Figure 2.5, Figure 2.6, and Figure 2.7). Furthermore, we demonstrated that constitutive activation of Foxo1 in liver was sufficient to decrease albumin gene expression (Figure 2.8). Taken together, we conclude that insulin stimulates albumin production by inhibiting Foxo1, which represses albumin expression.

Interestingly, even though concomitant deletion of *Foxo1* completely restored the reduced albumin gene expression in AktDKO livers, there remained a small, yet statistically significant decrease in albumin protein level in serum, suggesting that insulin may also regulate albumin production at a post-transcriptional site downstream of Akt. To this end, we investigated the role of mTORC1, a complex known to stimulate protein translation downstream of insulin, in serum albumin production.

Impaired mTORC1 activity in AktDKO liver also contributed to decreased albumin production because when we restored mTORC1 activity by deleting *Tsc1* in addition to *Akt1 and Akt2* specifically in the liver, serum albumin level increased from 50% of control level to 75% of control (Figure 2.9B). mTORC1 contributed to albumin production on a post-transcription level, as albumin gene

expression was not affected by restoring hepatic mTORC1 activity (Figure 2.9C). Taken together, these results suggest that downstream of Akt, albumin gene expression is controlled by Foxo1, whereas mTORC1 may contribute to post-transcriptional production of serum albumin, presumably on the level of protein translation and/or secretion. Similarly, disrupting mTORC1 activity by deleting *Raptor* specifically in the liver had no effect on albumin gene expression (Figure 2.10C). Surprisingly, serum protein level was not significantly reduced in the absence of hepatic mTORC1 activity (Figure2.10B). At first glance, results in TSC1TKO and RaptorKO livers seem to be inconsistent with regard to whether mTORC1 contributes to serum albumin production. We speculate that normal serum albumin level in RaptorKO animals might be the result of elevated hepatic autophagy, which also contributes to albumin production (see below). Nevertheless, mTORC1 exerts a relatively minor effect on serum albumin production, suggesting that regulation of albumin transcription plays a more dominant role than translational regulation in serum albumin production.

Autophagy is an important physiological process that maintains energy balance, disposes misfolded proteins, and removes damaged cellular compartments. Autophagy is activated by increased cellular stress and inhibited by activation of mTORC1 (Rabinowitz and White, 2010; Yamada and Singh, 2012). As a result of dampened mTORC1 activity in AktDKO livers, autophagy was elevated, as indicated by the decreased level of p62, a protein degraded by autophagy (Figure 2.11). We asked whether increased autophagy contributed to the reduced production of albumin observed in AktDKO animals.

Our results indicate that in postprandial state, autophagic activity is low and exerts little effect on albumin production. On the other hand, we observed a small yet statistically significant decrease in serum protein level in overnight fasted Atg5KO animals (Figure 2.12E), suggesting that opposite to what we expected, hepatic autophagy, a catabolic process, positively contributes to serum albumin production. Furthermore, this level of regulation occurred at a post-transcriptional site, as disruption of hepatic autophagy did not affect albumin gene expression level (Figure 2.12F).

Deletion of *Atg5* in addition to *Akt1* and *Akt2* specifically in the liver (Atg5TKO) led to the development of severe edema. Atg5TKO mice accumulated significant amount of fluid (equivalent to a third of body weight) in the interstitial space (Figure 2.13B-E), suggesting that oncotic pressure in these animals was severely dysregulated. Atg5TKO animals exhibited normal liver weight compared to controls (Figure 2.13F). Since Akt-null livers were smaller than controls, this implies that disruption of hepatic autophagy in this model also induced hepatomegaly. Consistent with the edema phenotype, serum protein of Atg5TKO animals was significantly reduced compared to controls (Figure2.14A). Interestingly, this reduction was exacerbated compared to AktDKO animals (~70% reduction vs. ~50% reduction, respectively), suggesting that activation of autophagy in AktDKO livers maintained some albumin production. This is consistent with our previous observation, where activation of hepatic autophagy during fasting positively contributed to albumin production.

How hepatic autophagy contributes to albumin production is currently unclear. One possible mechanism is that autophagy generates substrates intracellularly to supply protein synthesis, since amino acids released from autophagic degradation can potentially be recycled for protein production (Narita et al., 2011; Rabinowitz and White, 2010). In addition, it is possible that the products of autophagic degradation are used for energy generation to support hepatic protein production (He et al., 2012; Rabinowitz and White, 2010; Singh et al., 2009b). It has been demonstrated recently that autophagy is also involved in both conventional and unconventional secretory pathways. Notably, in senescent cells, autophagosomes co-localize with mTORC1 to form the TOR-autophagy spatial coupling compartment (TASCC). This co-localization allows for spatial coupling of autophagic degradation, which generates a high flux of substrates, directly with mTORC1-mediated biosynthesis for efficient synthesis and secretion of proteins in these cells (Narita et al., 2011). It is possible that such compartment also exists in liver, and autophagy contributes to albumin production by augmenting its secretion.

Somewhat surprisingly, we also observed disconnect between serum albumin level and albumin gene expression in Atg5TKO animals. Specifically, even though serum protein levels in these animals were dramatically decreased, hepatic albumin mRNA was slightly increased compared to controls (Figure 2.14B). We speculate this is the result of the feedback mechanism in response to the drastic decrease in oncotic pressure, the main regulator of albumin synthesis (Fanali et al., 2012; Nicholson, 2000). It has been shown that

intravenous infusion of macromolecules in rats can decrease the transcriptional activity of albumin to compensate for the increase in oncotic pressure (Pietrangelo et al., 1992). In Atg5TKO animals, oncotic pressure was significantly reduced as a result of severely reduced albumin production, and it is possible that albumin transcription was stimulated as a compensatory mechanism. It is important to note that under this model, oncotic pressure regulates albumin transcription by a pathway independent of Akt.

In contrast to what was observed in STZ-induced Type 1 diabetic animals, serum albumin production and transcription are slightly elevated in leptin-deficient animals, suggesting differential regulation of albumin by insulin in Type 1 versus Type 2 diabetes. Consistent with this result, studies in human patients also showed that serum albumin production is not affected by Type 2 diabetes (Tessari et al., 2006b). The increased albumin expression and protein level in leptin-deficient animals are likely the compensatory induction as a result of albuminuria in these animals (Hudkins et al., 2010; Tessari et al., 2006a). Alternatively, our result suggests that insulin action on serum albumin production is maintained during insulin resistance, and the increased albumin gene expression could be the result of hyperinsulinemia in these animals. During insulin resistance, insulin's ability to stimulate glucose uptake and to inhibit hepatic glucose production is impaired, yet insulin continues to stimulate *de novo* lipogenesis, resulting hypertriglyceridemia. Currently, there are two prevailing views to explain the paradoxical pathology of metabolic disease. Under the model of "selective insulin resistance", only specific metabolic pathways are

resistant to insulin while the others remain intact: insulin fails to suppress hepatic glucose production, while continuing to sustain hepatic lipogenesis (Brown and Goldstein, 2008). It is possible that in Type 2 diabetes, the regulation of insulin on albumin production also remains intact. An alternative model suggests that rather than impaired insulin action, the metabolic defects are mainly caused by altered substrate delivery and nutrient handling (Otero et al., 2014). Under this model, our result would imply that transcription and secretion of serum albumin is unaffected by altered nutrient flux to the liver during insulin resistance.

Figures

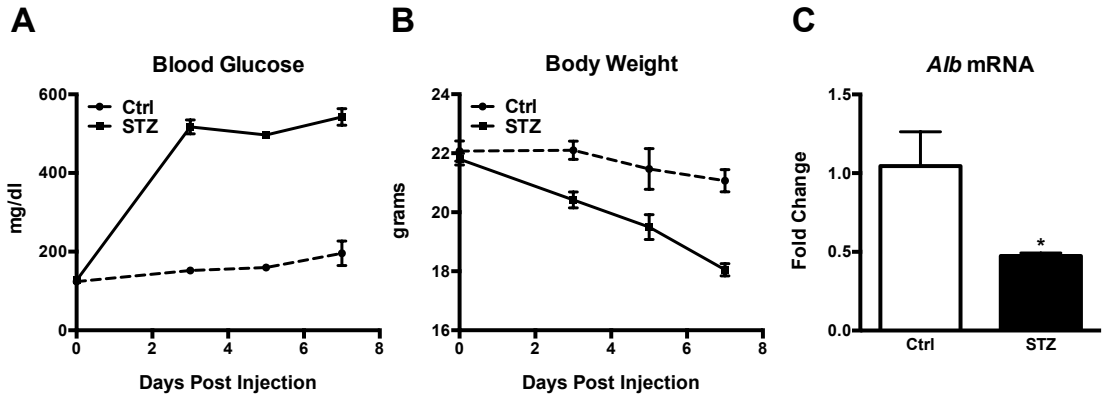


Figure 2.1: Albumin expression is decreased in Type 1 diabetic livers.

A,B. (A) Blood glucose and (B) body weight of mice that received an intra-peritoneal injection of either control buffer (Ctrl) or Steptozotocin (STZ) at 200mg per kg body weight.

C. Hepatic albumin mRNA level measured 9 days post STZ injection.

All values are expressed as mean ± SEM. n = 3-5; *p<0.05 vs. Ctrl by two-tailed Student's t-test.

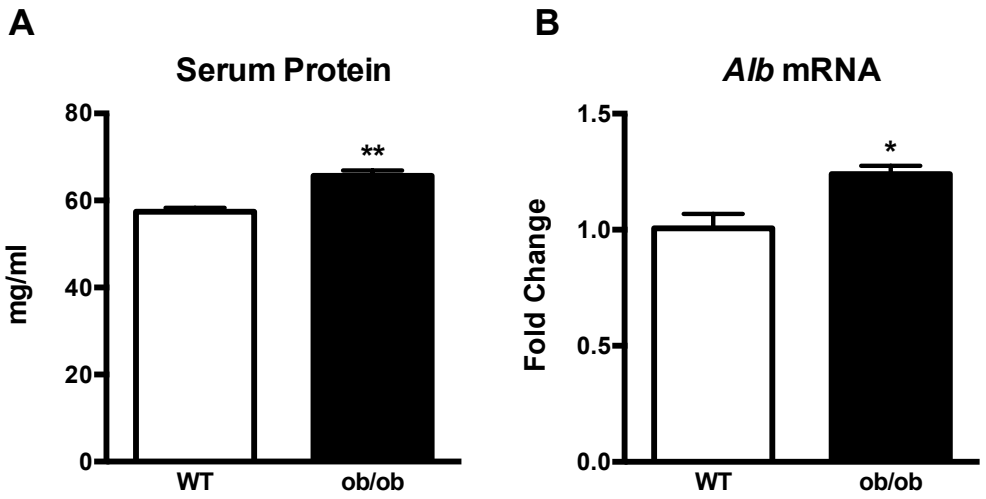


Figure 2.2: Albumin expression and production are normal in Type 2 diabetic livers.

A. Total protein concentration in serum of wildtype (WT) and leptin-deficient (*ob*/*ob*) mice.

B. Hepatic albumin mRNA levels of WT and *ob*/*ob* mice.

All values are expressed as mean ± SEM. n = 4-5; *p<0.05 vs. WT and **p<0.01 vs. WT by two-tailed Student's t-test.

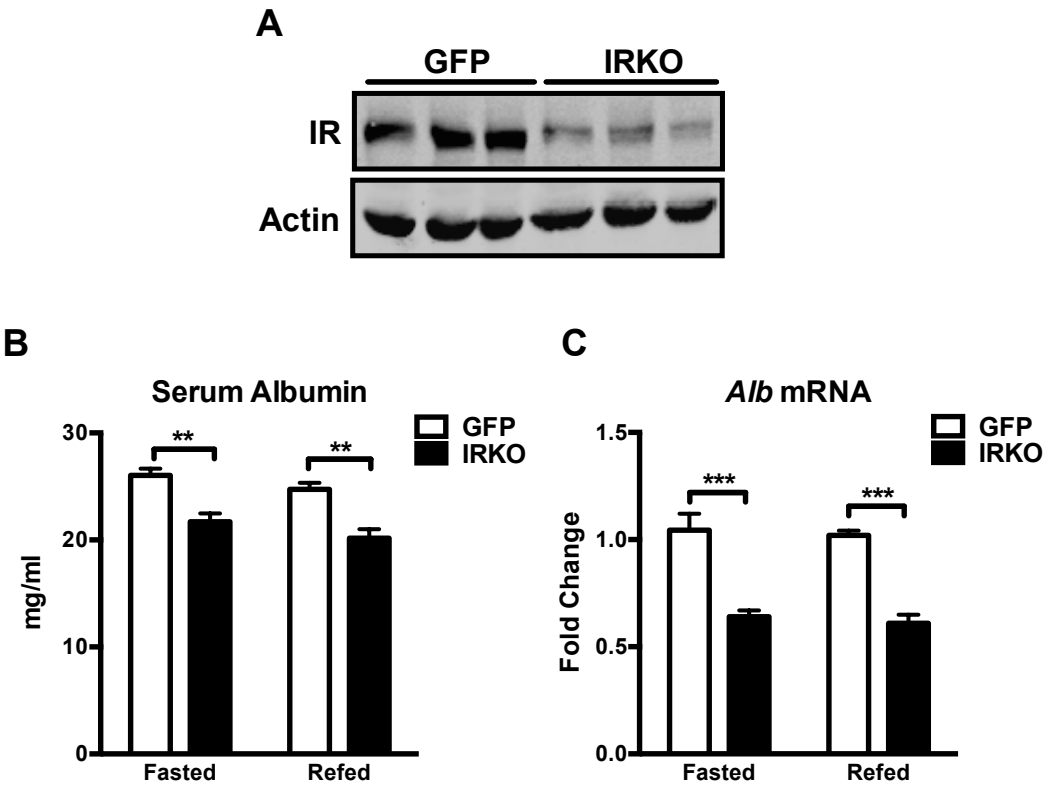


Figure 2.3: Insulin signals directly in the liver to stimulate albumin expression.

A. Western blots for insulin receptor (IR) and actin in liver homogenates of GFP control (GFP) and liver-specific *Ir* knockout (IRKO) animals.

B,C. Serum albumin concentration (B) and hepatic albumin mRNA level (C) in GFP and IRKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

All values are expressed as mean ± SEM. n = 3-6; **p<0.01 vs. GFP and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

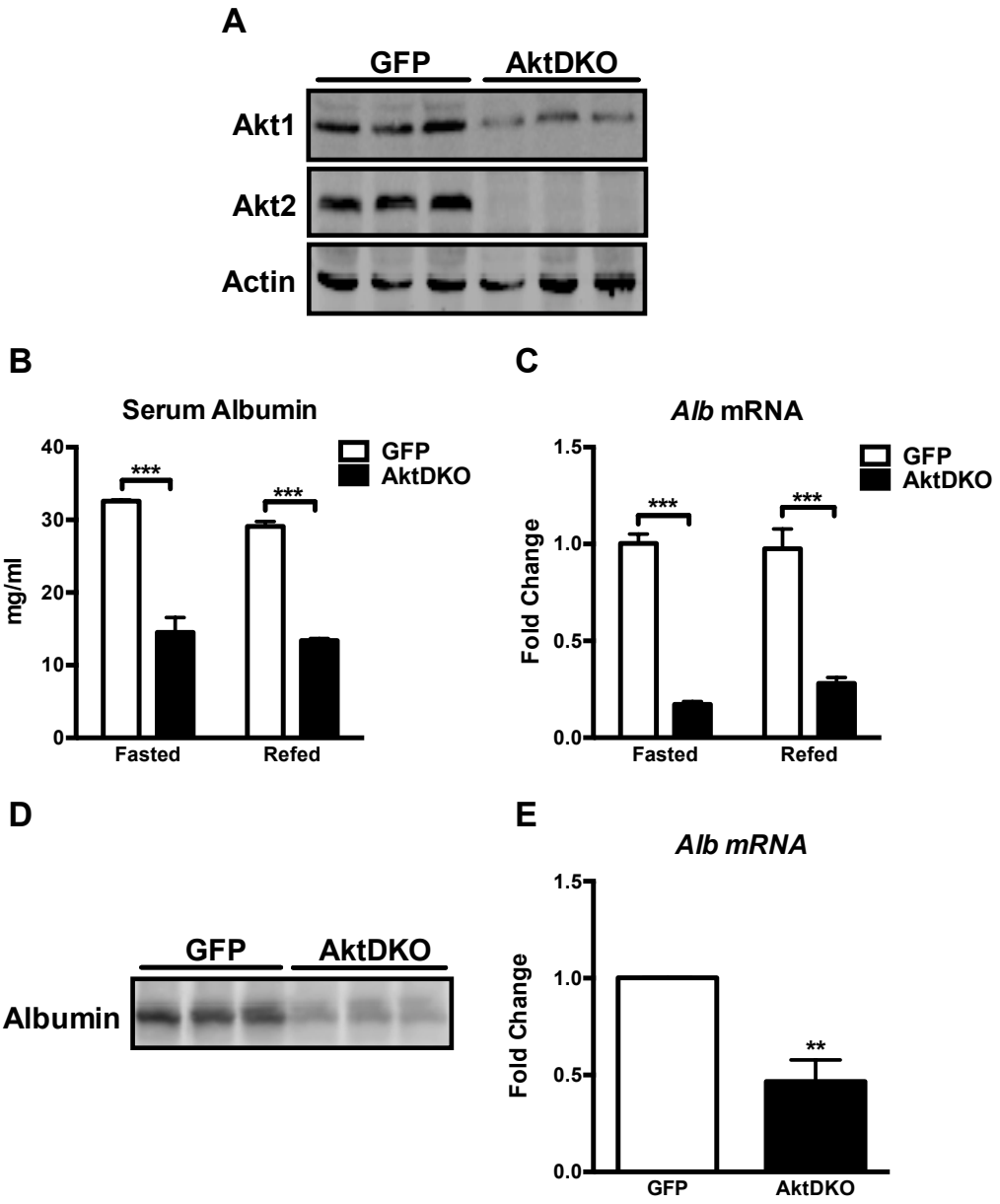


Figure 2.4: Akt is required to mediate insulin's effect on albumin production.

A. Western blots for Akt1, Akt2, and actin in liver homogenates of GFP control (GFP) and liver-specific *Akt1*/*Akt2* double-knockout (AktDKO) animals.

B,C. Serum albumin concentration (B) and hepatic albumin mRNA level (C) in GFP and AktDKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours. n = 3-4; **p<0.01 vs. GFP by two-way ANOVA using Sidak post-test.

D. A representative experiment of *in vitro* albumin secretion assay. Primary hepatocytes isolate from GFP and AktDKO livers were cultured in serum-free media for 2 hours. Secreted proteins were TCA precipitated and subjected to Western blot for albumin. Experiment was repeated 3 times.

E. Albumin mRNA level in primary hepatocytes isolated from GFP and AktDKO livers were assayed by RT-qPCR. n = 3-4; **p<0.01 vs. GFP by two-tailed Student's t-test.

All values are expressed as mean ± SEM.

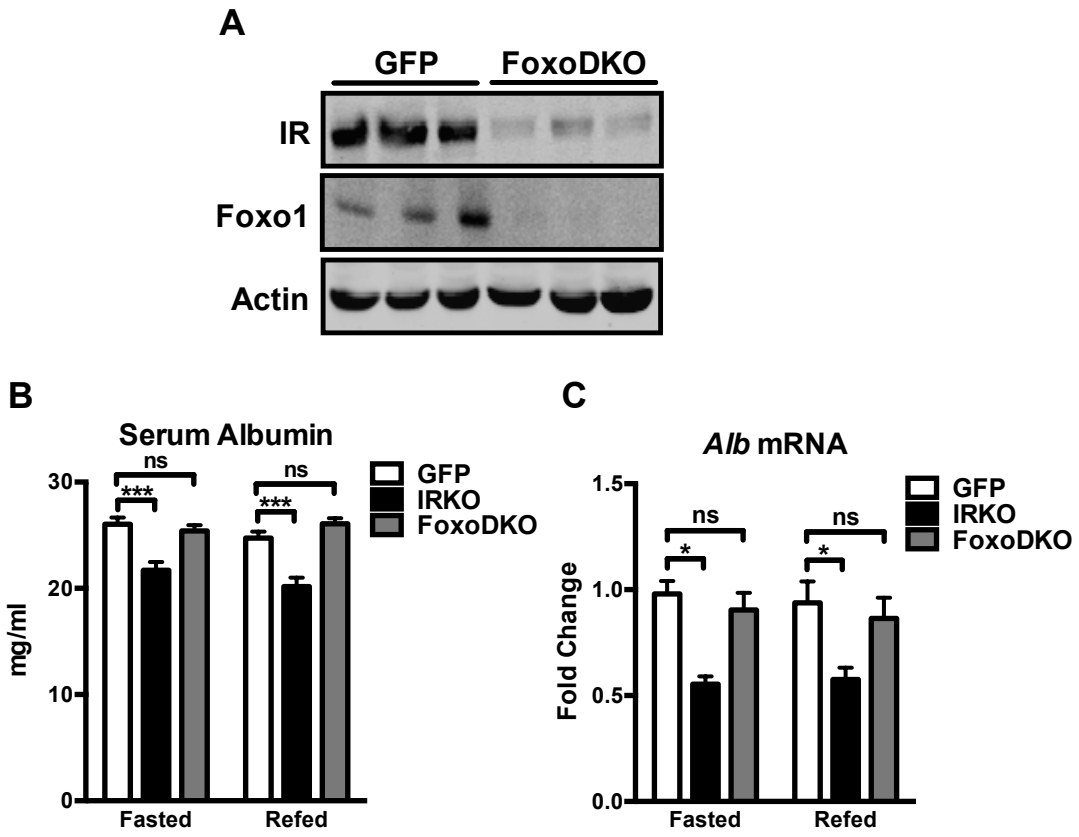


Figure 2.5: Inhibition of Foxo1 restores reduced albumin production in liver-specific insulin receptor knockout mice.

A. Western blots for insulin receptor (IR), Foxo1, and actin in liver homogenates of GFP control (GFP) and liver-specific *Ir*/*Foxo1* double-knockout (FoxoDKO) animals.

B,C. Serum albumin concentration (B) and hepatic albumin mRNA level (C) in GFP, liver-specific *Ir* knockout (IRKO), and FoxoDKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

All values are expressed as mean ± SEM. n = 4-5; ns, not significant, *p<0.05 vs. GFP, and ***p<0.001 vs. WT by two-way ANOVA using Sidak post-test.

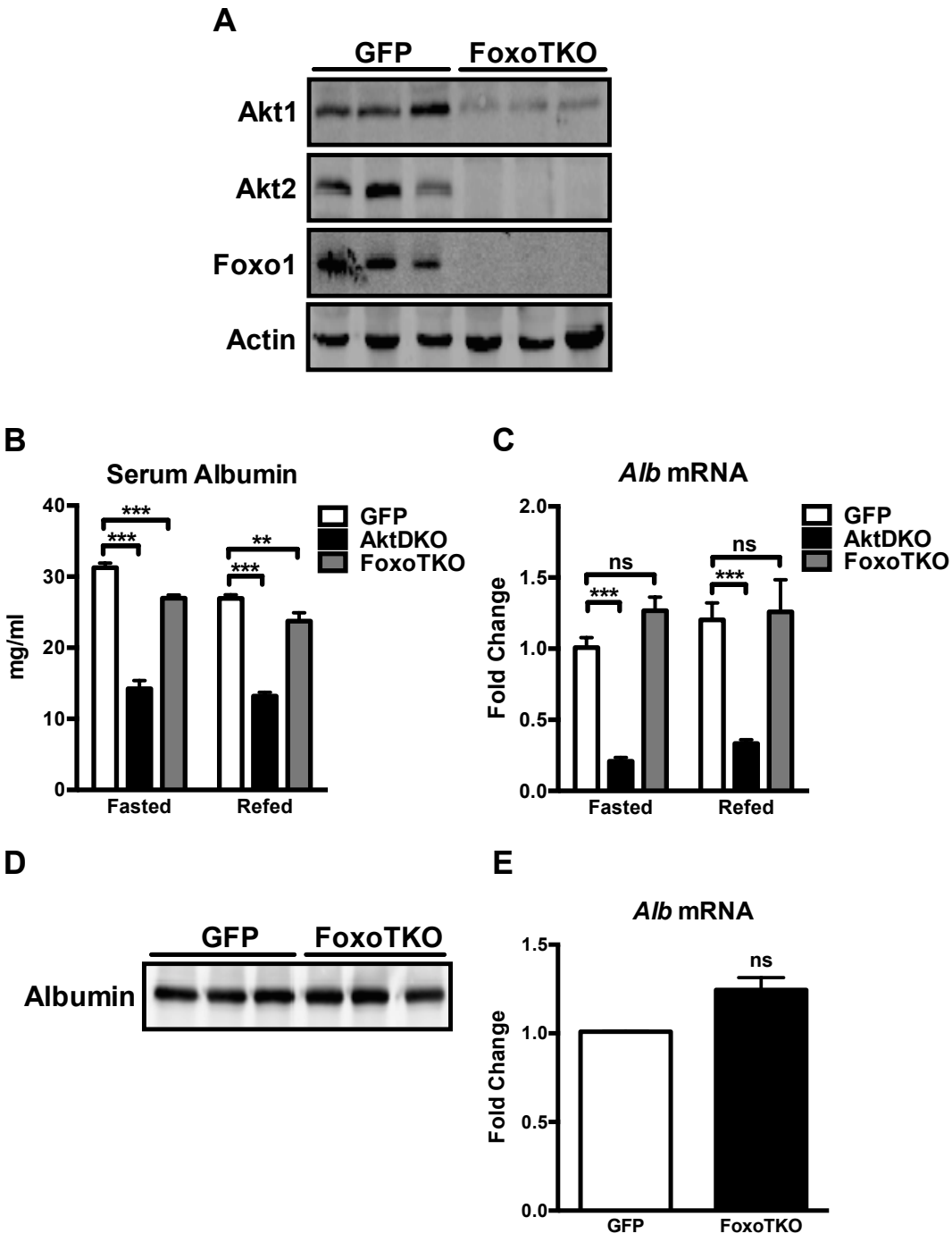


Figure 2.6: Inhibition of Foxo1 restores reduced albumin production in liver-specific *Akt1*/*Akt2* double-knockout mice.

A. Western blots for Akt1, Akt2, Foxo1, and actin in liver homogenates of GFP control (GFP) and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) animals.

B,C. Serum albumin concentration (B) and hepatic albumin mRNA level (C) in GFP, liver-specific *Akt1*/*Akt2* double-knockout (AktDKO), and FoxoTKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours. n = 3-5; ns, not significant, **p<0.01 vs. GFP, and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

D. A representative experiment of *in vitro* albumin secretion assay. Primary hepatocytes isolated from GFP and FoxoTKO livers were cultured in serum-free media for 2 hours. Secreted proteins were TCA precipitated and subjected to Western blot for albumin. Experiment was repeated 3 times.

E. Albumin mRNA level in primary hepatocytes isolated from GFP and FoxoTKO livers were measured by RT-qPCR. n = 3-4; ns, not significant by two-tailed Student's t-test.

All values are expressed as mean ± SEM.

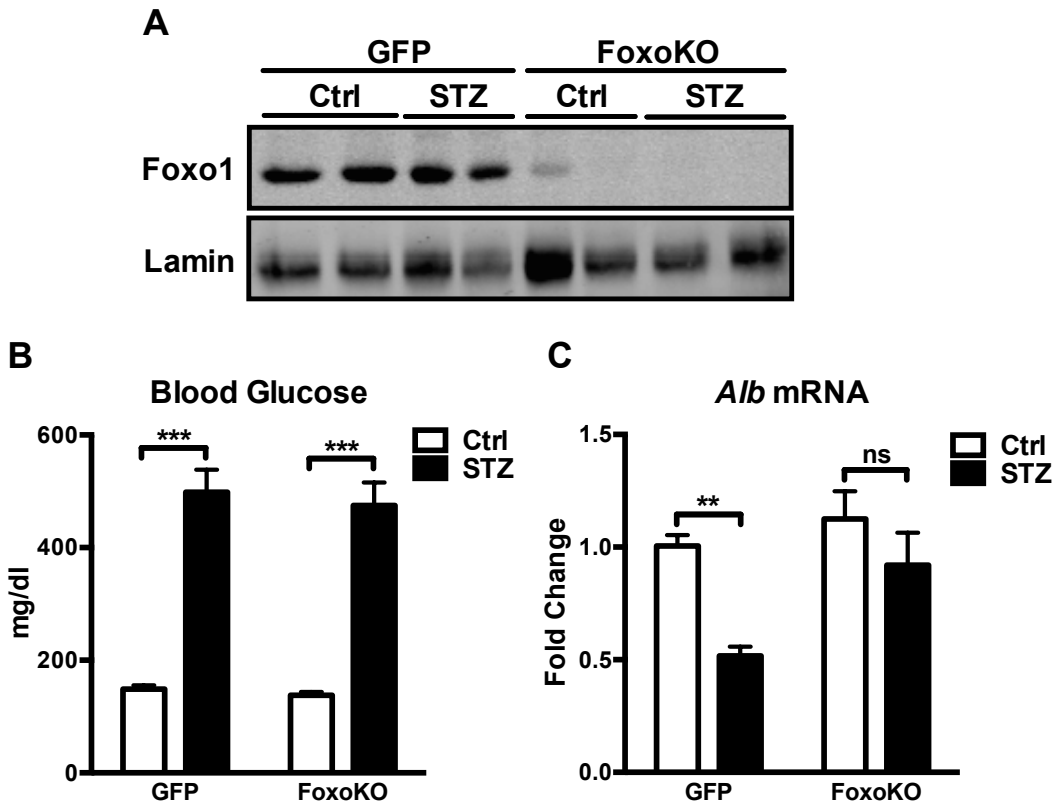


Figure 2.7: Inhibition of Foxo1 restores reduced albumin expression in streptozotocin-induced Type 1 diabetic livers.

A. Western blots for Foxo1 and lamin in liver nuclear extracts of GFP control (GFP) and liver-specific *Foxo1* knockout (FoxoKO) animals.

B,C. Blood glucose (B) and hepatic albumin mRNA level (C) of GFP and FoxoKO animals 9 days post an intra-peritoneal injection of either buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight.

All values are expressed as mean ± SEM. n = 5-7; ns, not significant, **p<0.01 vs. Ctrl and ***p<0.001 vs. Ctrl by two-way ANOVA using Sidak post-test.

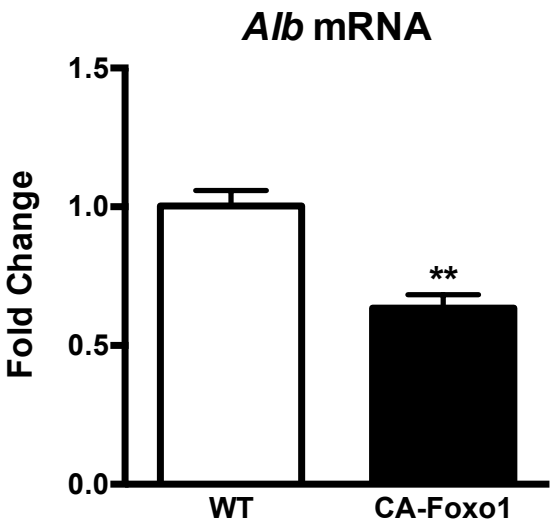


Figure 2.8: Activation of hepatic Foxo1 is sufficient to suppress albumin expression.

Hepatic albumin mRNA level of wildtype (WT) and liver-specific transgenic mice expressing a constitutively active Foxo1 (CA-Foxo1) were measured by RT-qPCR.

All values are expressed as mean ± SEM. n = 3; **p<0.01 vs. WT by two-tailed Student's t-test.

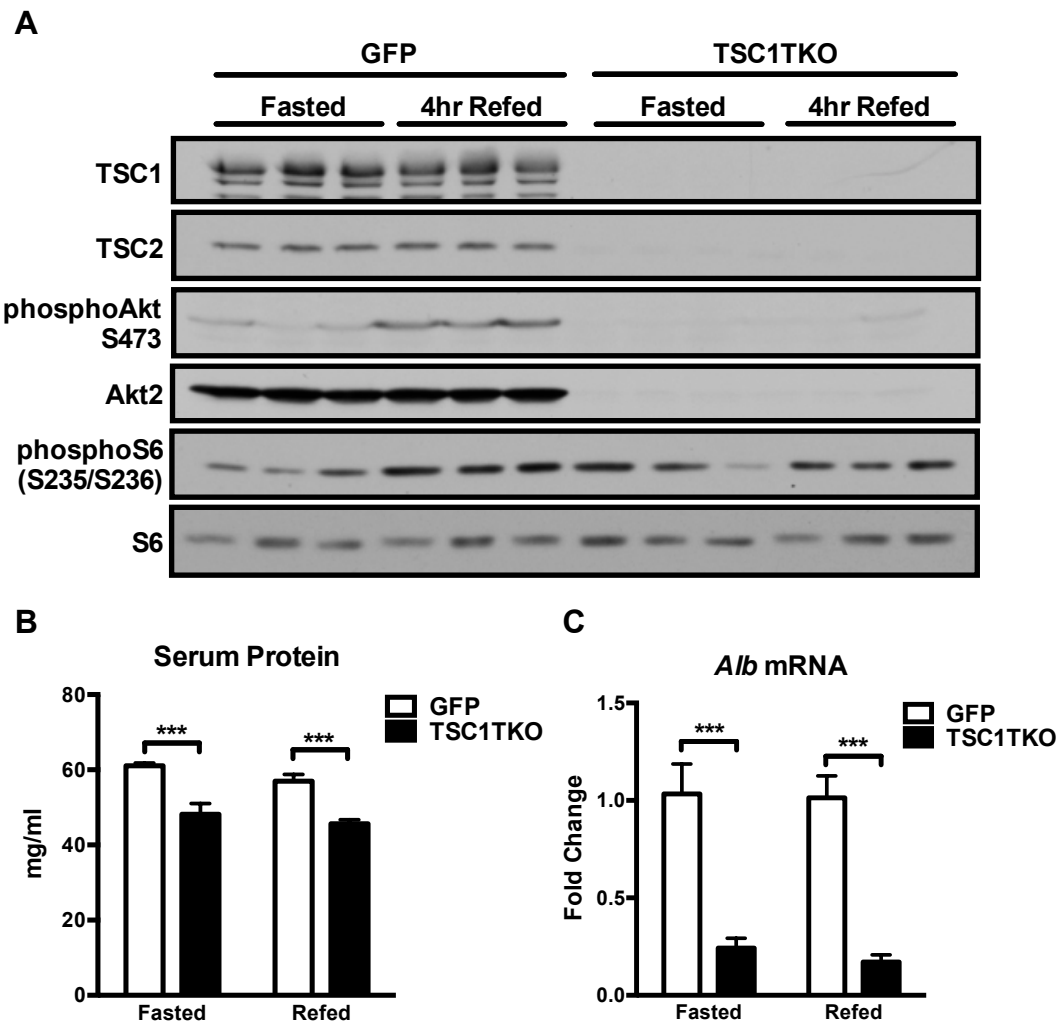


Figure 2.9: Activation of mTORC1 partially restores reduced albumin production in liver-specific *Akt1*/*Ak2* double-knockout mice with no effect on albumin gene expression.

A. Western blots for tuberculosis sclerosis complex (TSC) 1, TSC2, phosphorylated Akt (S473), Akt2, phosphorylated ribosomal protein S6 (S235/S236), and ribosomal protein S6 in liver homogenates of GFP control

(GFP) and liver-specific *Tsc1*/*Akt1*/*Akt2* triple-knockout (TSC1TKO) animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

B-D. Total protein concentration in serum (C) and albumin mRNA level (D) in GFP and TSC1TKO animals that had been either fasted overnight or fasted overnight and refed for 4 hrs.

All values are expressed as mean ± SEM. n = 4-5; ns, not significant and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

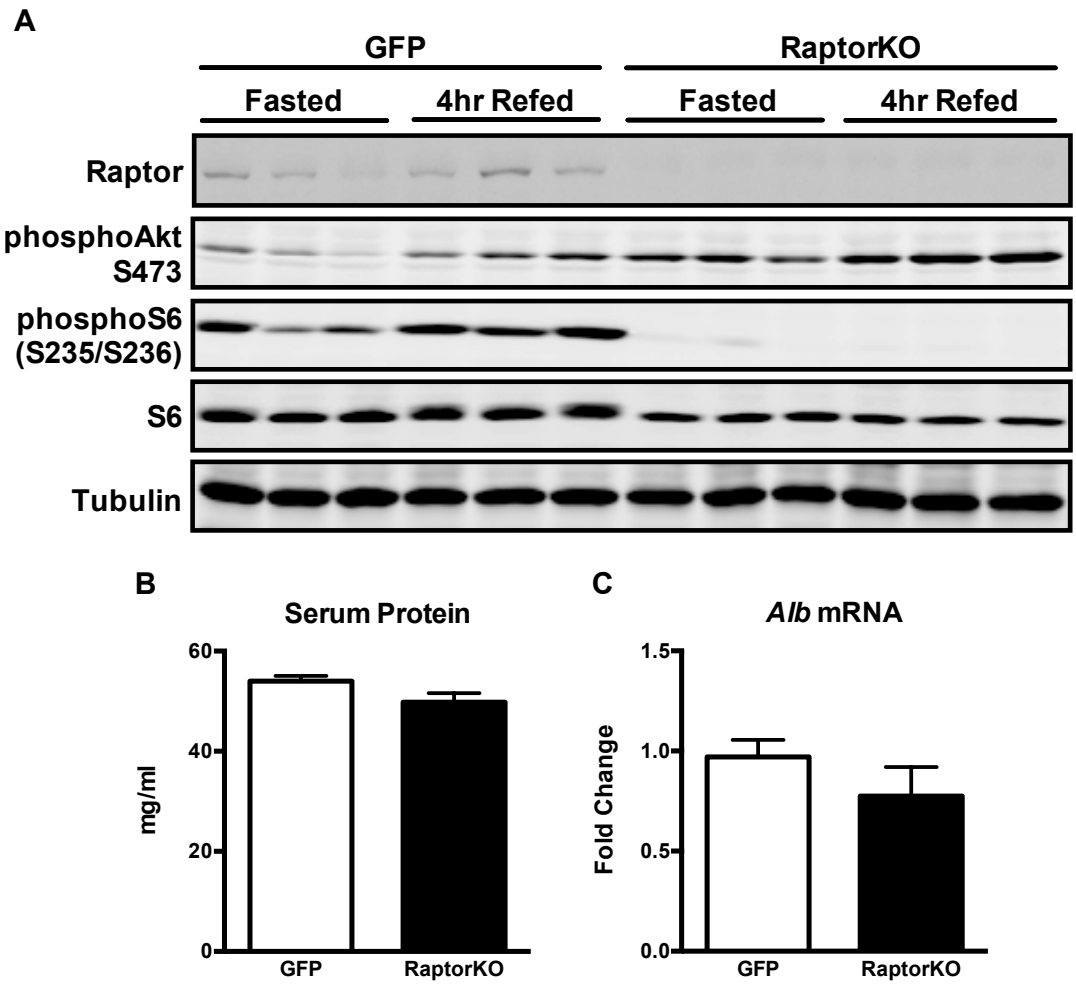


Figure 2.10: mTORC1 is not required for proper hepatic albumin production.

A. Western blots for Raptor, phosphorylated Akt (S473), phosphorylated ribosomal protein S6 (S235/S236), ribosomal protein S6, and tubulin in liver homogenates of GFP control (GFP) and liver-specific *Raptor* knockout (RaptorKO) animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

B,C. Total protein concentration in serum (B) and hepatic albumin mRNA level (C) of GFP and RaptorKO animals.

All values are expressed as mean ± SEM. n = 9; *p<0.05 vs. GFP and ***p<0.001 vs. GFP by two-tailed Student's t-test.

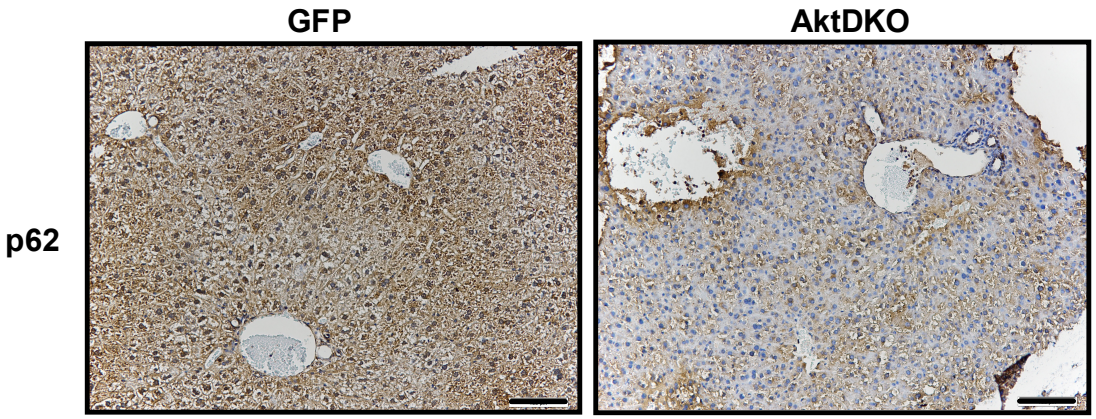


Figure 2.11: Disruption of Akt signaling in the liver leads to elevated hepatic autophagy.

Immunohistochemical staining for p62, a protein degraded by autophagy, in GFP control (GFP) and Akt-null (AktDKO) livers. Scale bars indicate 100μm.

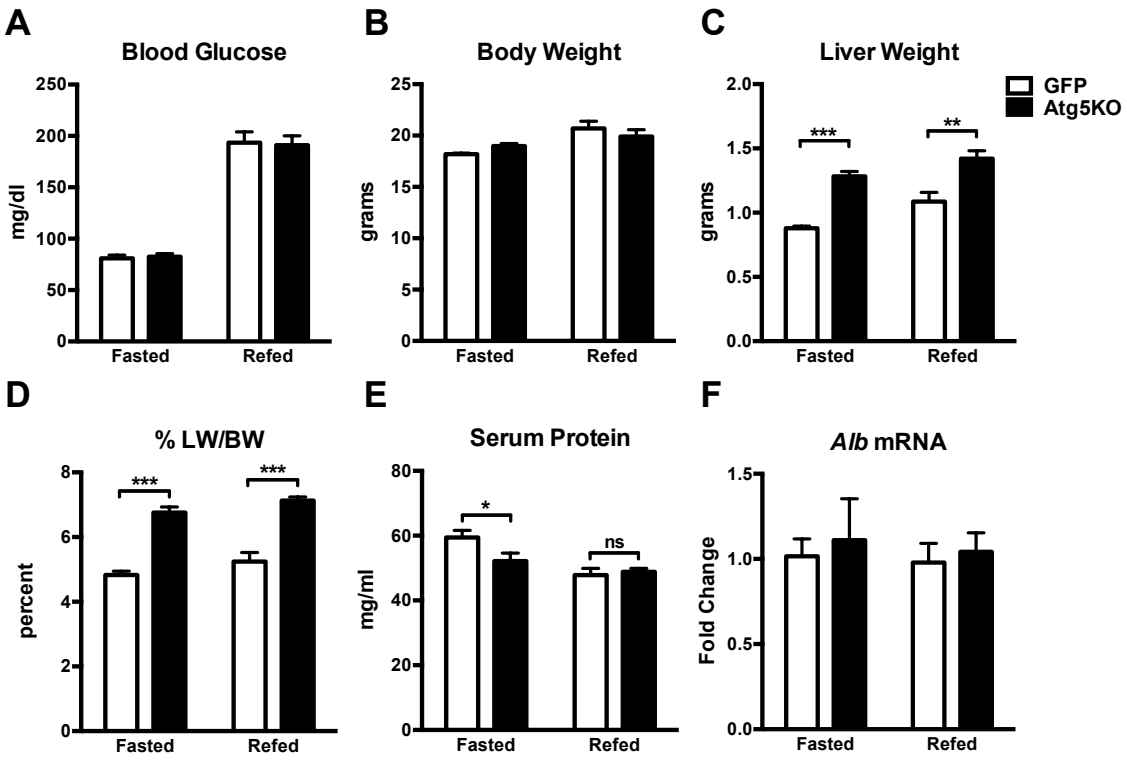


Figure 2.12: Disruption of hepatic autophagy in wildtype mice has no effect on albumin production.

Blood glucose (A), body weight (B), liver weight (C), liver weight as a percent of body weight (D), total protein concentration in serum (E), and hepatic albumin mRNA level (F) of GFP control (GFP) or liver-specific *Atg5* knockout (Atg5KO) mice that had been either fasted over night or fasted overnight and refed for 4 hours.

All values are expressed as mean ± SEM. n = 4-5; ns, not significant, *p<0.05 vs. GFP, **p<0.01 vs. GFP, and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

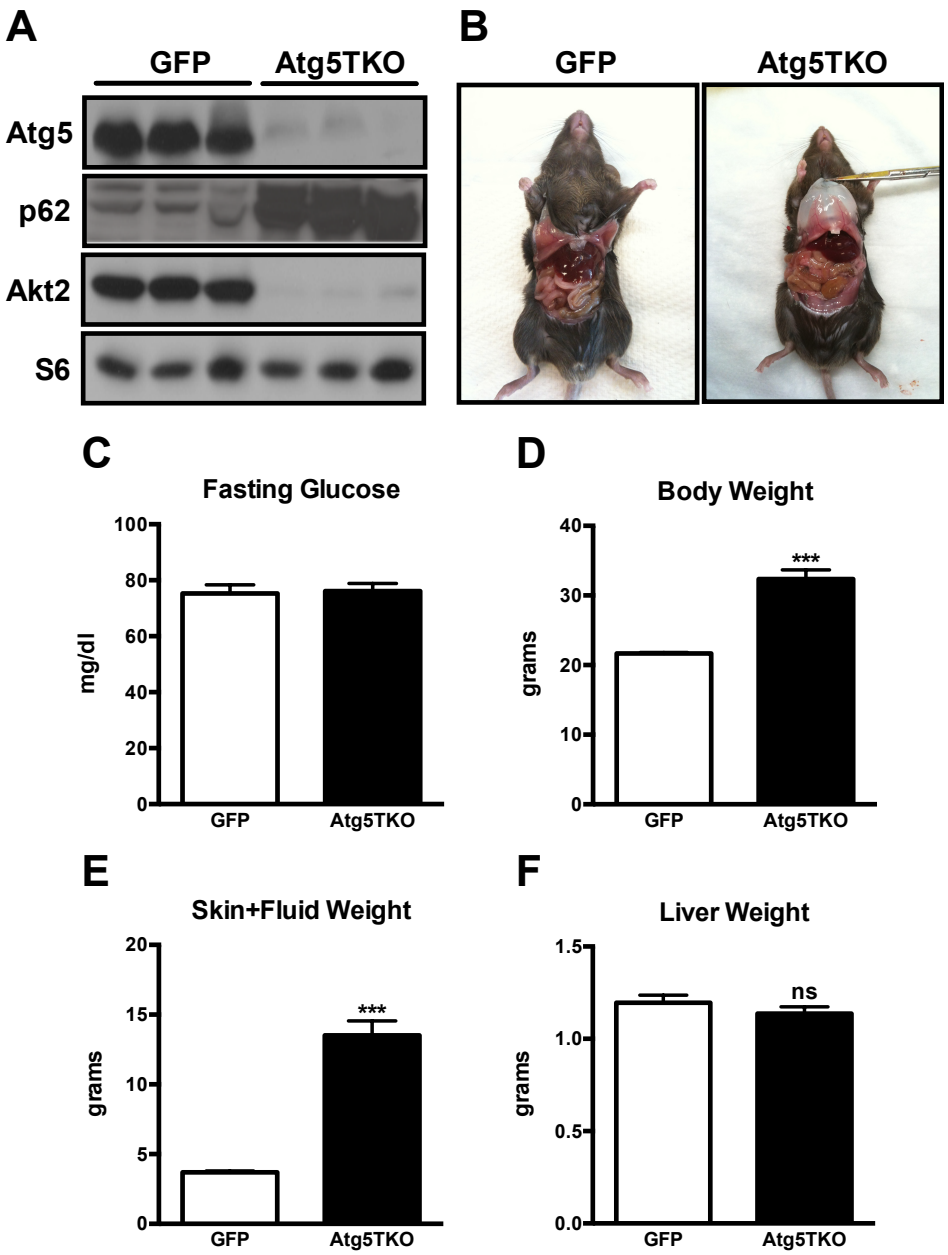


Figure 2.13: Disruption of hepatic autophagy in liver-specific *Akt1*/*Akt2* double-knockout mice causes severe edema.

A. Western blots for Atg5, p62, Akt2, and ribosomal protein S6 in liver homogenates of GFP control (GFP) and liver-specific *Atg5*/*Akt1*/*Akt2* triple-knockout (Atg5TKO) animals.

B. Gross appearance of the peritoneal cavity of GFP and Atg5TKO mice.

C-F. Fasting blood glucose (C), body weight (D), skin and fluid weight (E, obtained by subtracting the weight of dissected carcass from body weight), and liver weight (F) of GFP and Atg5TKO mice.

All values are expressed as mean ± SEM. n = 6-9; ns, not significant and ***p<0.001 vs. GFP by two-tailed Student's t-test.

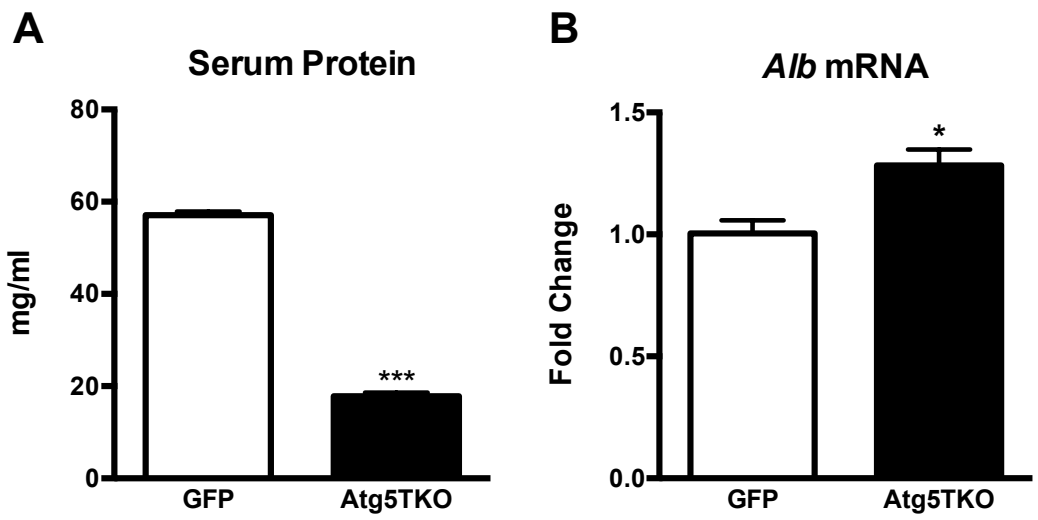


Figure 2.14: Disruption of hepatic autophagy in liver-specific *Akt1*/*Akt2* double-knockout mice restores reduced albumin expression but not reduced circulating albumin.

Total protein concentration in serum (A) and hepatic albumin mRNA level (B) in GFP control (GFP) and liver-specific *Atg5*/*Akt1*/*Akt2* triple-knockout (Atg5TKO) mice.

All values are expressed as mean ± SEM. n = 6-9; *p <0.05 vs. GFP and ***p<0.001 vs. GFP by two-tailed Student's t-test.

Chapter 3

Mechanisms of albumin gene regulation downstream of Foxo1

Introduction

Although the mechanism by which insulin regulates albumin transcription has not been elucidated until the present study, the transcriptional regulation of the albumin gene has been extensively characterized: Six distinct *cis* elements in the promoter region have been identified, as well as a handful of liver-enriched transcription factors that interact with the *Alb* promoter to activate albumin transcription, including hepatic nuclear factor 1α (Hnf-1α), D site binding protein (Dbp), and CCAAT/enhancer binding proteins (C/EBPs) (Lichtsteiner et al., 1987; Maire et al., 1989). Of interest, C/EBPs are key regulators for cell differentiation and proliferation (Nerlov, 2008). In liver, C/EBPs are essential for maintaining normal liver functions such as albumin production and bilirubin detoxification and clearance (Inoue et al., 2004; Y H Lee, 1997). In addition, C/EBPs control the expression of key gluconeogenic and lipogenic enzymes, thus playing a significant role in regulating glucose and lipid metabolism in liver as well.

In the previous chapter, we have demonstrated that insulin signals directly on the liver to inhibit Foxo1, which functions as a transcriptional repressor of the albumin gene. The specific mechanism of how Foxo1 functions as a repressor remains largely unknown. Studies in C. *elegans* show that genes downregulated by DAF-16, the Foxo ortholog, are not directly bound by DAF-16, suggesting that Foxo represses gene expression by an indirect mechanism (Ramaswamy et al., 2002; Tullet, 2014). A recent study described a transcription factor called PQM-1, which exhibits subcellular localization that is mutually antagonistic with that of DAF-16: When DAF-16 is active and nuclear, PQM-1 is excluded from the

nucleus and subsequently its target genes are downregulated. This provides a mechanism by which DAF-16 functions as a gene repressor (Tepper et al., 2013). To date, it is unclear whether an orthologous mechanism exists in mammals. Nevertheless, recent studies in mammalian systems have provided evidence that Foxo1 could potentially repress gene expression by inducing the expression of other transcriptional repressor(s), or modulating the activity of other transcription factors.

In this chapter, we interrogated possible mechanisms by which Foxo1 represses albumin gene expression. We found that it is unlikely that Foxo1 induces the expression of small heterodimer partner (Shp) or inhibitor of DNA binding protein 3 (Id3) to repress the albumin gene. Interestingly, the transcriptional activity of C/EBPα was negatively correlated with Foxo1 activity. Furthermore, using chromatin immunoprecipitation (ChIP), we found that constitutive activation of Foxo1 was correlated with reduced C/EBPα binding to the albumin promoter.

Results

Shp and Id3 are unlikely the transcriptional repressors for albumin gene expression downstream of Foxo1

We first assessed the model in which Foxo1 functions as a repressor indirectly by inducing the expression of a transcriptional repressor, which in turn represses target genes. Shp and Id3 are transcription repressors that have been

identified as putative targets of Foxo1 (Shin et al., 2012; Wei et al., 2011). Moreover, previous studies have suggested connections between these factors and albumin expression (Nakayama et al., 2006; Park et al., 2007). To test whether Shp or Id3 represses albumin transcription downstream of Foxo1, we first measured their gene expression in our genetic models. We found that for both *Shp* and *Id3*, gene expression was significantly induced in Akt-null livers where Foxo1 was constitutively active, and concomitant deletion of *Foxo1* completely reversed the gene induction (Figure 3.1A and B). This observation is consistent with previous studies that Foxo1 directly induces Shp and Id3 transcription. In addition, the reciprocal expression pattern between albumin and *Shp* and *Id3* is consistent with the model where Shp and/or Id3 repress albumin expression. Nevertheless, the postprandial changes in *Shp* and *Id3* gene expression in control animals are inconsistent with the model where Foxo1 induces the expression of these repressors. Specifically, upon feeding when Foxo1 becomes inhibited by insulin, *Shp* and *Id3* expression should be downregulated according to the model. Yet, our data shows that *Shp* expression was unchanged and *Id3* expression was induced (Figure 3.1A and B), suggesting that Foxo1 might not be a physiologic regulator of *Shp* and *Id3.*

In addition, in *Ir*-null livers and streptozotocin (STZ)- induced diabetic livers, where Foxo1 was also constitutively active, we did not observe an induction of *Shp* or *Id3* gene expression (Figure 3.1C-F). This inconsistency suggests that the regulation of *Shp* and *Id3* is more complex. Therefore, it is

unlikely that Foxo1 represses albumin gene expression by inducing these transcription repressors.

Gene expression of Cebpa *was reduced in Akt-null livers*

To investigate whether Foxo1 represses albumin expression by directly affecting the activity of a transcription factor, we then shifted our attention to the transcription factors known to regulate albumin expression. Distinct *cis*-regulatory sites have been identified in the *Alb* promoter, as well as the transcription factors bound at each site (Lichtsteiner et al., 1987; Maire et al., 1989)(Figure 3.2A). Notably, all factors identified are transcription activators. We decided to focus on examining C/EBPα, C/EBPβ, Dbp, and Hnf-1α because these transcription factors are liver-enriched and are associated with binding sites with the strongest activating potential (Maire et al., 1989).

We first measured the hepatic gene expression levels of these transcription factors. Interestingly, we found that *Cebpa* showed a significant decrease in gene expression when *Ir* was specifically deleted in the liver (IRKO) in the fasted state and exhibited a trend of decrease that was not statistically significant in the fed state (Figure 3.2A), while the gene expression of *Cebpb*, *Dbp*, and *Hnf1a* was not affected (Figure 3.2B-E). More importantly, concomitant deletion of *Foxo1* (FoxoDKO) fully restored the decreased *Cebpa* expression to control levels (Figure 3.2B). Similarly, liver-specific deletion of *Akt1* and *Akt2* (AktDKO) had no effect on the expression levels of *Cebpb*, *Dbp*, and *Hnf1a*, but

led to a significant decrease in *Cebpa* gene expression (Figure 3.3A-D). Concomitant deletion of *Foxo1* completely restored the decreased *Cebpa* expression to control levels in the fasted state, but only partially in the fed state (Figure 3.3A). These results show that under certain conditions, *Cebpa* gene expression exhibits the same pattern as albumin, and both are negatively correlated with hepatic Foxo1 activity. In addition, there seems to be other nutrient-dependent, Foxo1-independent pathways that regulate *Cebpa* expression.

We then asked whether the gene expression levels of these transcription factors were differentially regulated in Type 1 diabetic livers as well and whether Foxo1 activity also contributed to the transcription regulation. To this end, we used STZ to induce diabetes in either control (GFP) or liver-specific *Foxo1* knockout mice (FoxoKO). Surprisingly, STZ treatment did not have any effect on the gene expression of these transcription factors (Figure 3.4A-D), despite of the elevated Foxo1 activity. Ablation of *Foxo1* also showed no effect, suggesting that the transcriptional regulation of these genes was independent of Foxo1.

To test whether the decreased *Cebpa* gene expression correlated with a decrease in protein level, we measured the hepatic C/EBPα protein levels by Western blotting. Interestingly, despite of the decreased mRNA levels, C/EBPα protein remained unchanged in IRKO and AktDKO livers, and concomitant deletion of *Foxo1* in these models had no effect on the C/EBPα protein levels (Figure 3.5A and Figure 3.6A, respectively). On the contrary, C/EBPβ protein levels were slightly increased in IRKO and AktDKO livers compared to control,

and concomitant ablation of *Foxo1* in these models normalized the C/EBPβ protein to control levels (Figure 3.5B and Figure 3.6B, respectively).

C/EBPα activity is negatively correlated with Foxo1 activity in liver

C/EBPα activates its own transcription in a positive feedback loop (Nerlov, 2008). Having observed a decreased *Cebpa* expression without detectable changes in C/EBPα protein levels, we hypothesized that C/EBPα activity was reduced in liver when Foxo1 was constitutively active. To test this idea, we set out to measure expression levels of the C/EBPα target genes.

11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1) is a direct target gene of C/EBPα (Inoue et al., 2004; Williams et al., 2000). Interestingly, we found that *Hsd11b1* gene expression was significantly decreased in AktDKO livers, and concomitant deletion of *Foxo1* fully rescued the expression to control levels (Figure 3.7A). This observation is consistent with the hypothesis that C/EBPα activity is negatively correlated with Foxo1 activity in the liver. To test whether C/EBPα activity was also reduced in Type 1 diabetic livers, we measured *Hsd11b1* mRNA level in control and STZ-induced diabetic livers. Development of diabetes did not affect hepatic *Hsd11b1* gene expression, suggesting that C/EBPα activity was unaffected in these livers. In addition, deletion of *Foxo1* specifically in the liver in either control or STZ-treated mice exhibited no effect on hepatic *Hsd11b1* gene expression (Figure 3.7B). These results suggest that C/EBPα activity was independent of Foxo1 activity in STZ-

induced Type 1 diabetes model. The discrepancy could be due to differences in experimental systems used (See Discussion).

We also investigated whether the negative correlation between Foxo1 activity and C/EBPα activity was a general phenomenon for all C/EBPα target genes or one specific for the regulation of *Alb* and *Hsd11b1*. To this end, we first compiled a list of potential C/EBPα target genes by identifying the overlap between the C/EBPα ChIP-seq dataset and the list of genes differentially regulated in *Cebpa* knockout mice (Jakobsen et al.; Pedersen et al., 2007). We reasoned that these genes would most likely represent the direct targets of C/EBPα as their expression levels are C/EBPα-dependent and they have C/EBPα bound near their transcription start sites. We then measured the mRNA levels of these identified genes in GFP, AktDKO, and FoxoTKO livers. Most of these genes exhibited differential expression in AktDKO livers compared to control. Interestingly, concomitant deletion of *Foxo1* (FoxoTKO) completely restored the altered gene expression back to control levels (Figure 3.7C). These results supported our hypothesis that it is a general mechanism where constitutive activation of Foxo1 is correlated with a decrease in C/EBPα activity.

Constitutive activation of Foxo1 is correlated with decreased C/EBPα and C/EBPβ occupancy at the albumin promoter

Recent studies suggest that Foxo1 directly interacts with C/EBPα in adipocytes and neonatal liver to modulate C/EBPα activity (Qiao and Shao, 2006;

Sekine et al., 2007). In addition, Foxo1 has been known to interact with transcription factors to interfere with their DNA binding (Deng et al., 2012; Dowell, 2003; Fan et al., 2009). To test whether constitutive activation of Foxo1 interferes with C/EBPα DNA binding at the albumin promoter, we performed chromatin immunoprecipitation (ChIP) of C/EBPα and C/EBPβ in control, AktDKO, and FoxoTKO livers. Figure 3.8A shows the C/EBPβ binding peaks identified by ChIP-seq in wildtype mouse livers at the albumin promoter. Since C/EBPα and C/EBPβ often bind to DNA as a heterodimer, the peaks were used to predict C/EBPα binding sites, which were confirmed by ChIP using an antibody directed against C/EBPα (Figure 3.8B). Interestingly, C/EBPα and C/EBPβ occupancy at the albumin promoter was reduced in AktDKO livers compared to controls, and concomitant deletion of *Foxo1* specifically in the liver reversed this decrease (Figure 3.8B and C). This result indicates that Foxo1 activity was negatively correlated with C/EBPα and C/EBPβ binding to the albumin promoter, possibly by directly interacting with them and interfering with their DNA binding.

Next, we investigated whether C/EBPα binding to the albumin promoter was also decreased in diabetic livers. To this end, we injected STZ to induce Type 1 diabetes in either control or FoxoKO mice and used ChIP to measure C/EBPα and C/EBPβ occupancy at the albumin promoter. In STZ-induced diabetic livers, C/EBPα binding to the albumin promoter was decreased compared to control only at Site 2 (approximately 2.5kb upstream of the transcription start site, Figure 3.9A). C/EBPβ binding to the albumin promoter, on the other hand, was increased in STZ-induced diabetic livers compared to control

(Figure 3.9B). Importantly, ablation of *Foxo1* restored all diabetes-induced changes in C/EBPα and C/EBPβ enrichment (Figure 3.9A and B). Taken together, these results suggest that development of diabetes alters the binding of C/EBPα and C/EBPβ to the albumin promoters in a Foxo1-dependent manner.

C/EBPα and C/EBPβ occupancy at the promoter of other Cebpa target genes

Since we observed decreased gene expression of other C/EBPα target genes such as *Cebpa* and *Hsd11b1* (Figure 3.3A and Figure 3.7A, respectively), we hypothesized that this is due to decreased C/EBPα binding to the promoter of these genes as well, and C/EBPα occupancy at these sites would also be negatively correlated with Foxo1 activity. To test this hypothesis, we first measured C/EBPα and C/EBPβ occupancy at the promoter of *Cebpa* (Figure 3.10A) using ChIP. C/EBPα showed a slightly decreased enrichment in AktDKO livers compared to control, although the difference did not reach statistical significance. Importantly, concomitant deletion of *Foxo1* restored the slight decrease back to control levels (Figure 3.10B). C/EBPβ binding to the promoter of *Cebpa* was not different among GFP, AktDKO, and FoxoTKO livers (Figure 3.10C). Next, we examined the occupancy of C/EBPα and C/EBPβ at the *Cebpa* promoter in STZ-induced diabetic livers. C/EBPα binding to the *Cebpa* promoter was not affected by the development of Type 1 diabetes (Figure 3.10D). C/EBPβ occupancy, however, showed an increase in diabetic livers compared to controls. Furthermore, this increase was abrogated by deletion of *Foxo1* specifically in the

liver (Figure 3.10 E), suggesting that increased binding of C/EBPβ to the *Cebpa* promoter was dependent on Foxo1 activity.

We also measured C/EBPα and C/EBPβ occupancy at the *Hsd11b1* promoter (Figure 3.11A). Similar to what we observed at the *Cebpa* promoter, we found a slight, yet statistically insignificant decrease in C/EBPα occupancy at the *Hsd11b1* promoter in AktDKO livers, which was restored when *Foxo1* was concomitantly deleted (Figure 3.11B). C/EBPβ binding to the *Hsd11b1* was slightly increased in AktDKO livers. However, this increase was independent of Foxo1 activity, as deletion of *Foxo1* showed no effect (Figure 3.11C). In STZ-induced diabetic livers, C/EBPα binding to the *Hsd11b1* promoter showed no difference when compared to controls, whereas C/EBPβ exhibited significantly increased enrichment, which was restored to control levels with the deletion of *Foxo1* (Figure 3.11D and E), suggesting that the increased C/EBPβ binding to these sites was dependent on Foxo1 activity.

Overexpressing C/EBPα in AktDKO livers to rescue the reduced albumin expression

Since we observed that the occupancy of C/EBPα at the albumin promoter directly correlated with albumin gene expression in GFP, AktDKO, and FoxoTKO livers, we hypothesized that the decreased C/EBPα DNA binding as a result of the constitutive activation of Foxo1 contributed to the decreased albumin expression. To test this model, we overexpressed C/EBPα in AktDKO mice to

see if this could rescue the reduced albumin gene expression. Using an adeno-associated virus, we achieved an approximately 4-fold overexpression of *Cebpa* in liver (Figure 3.12B). C/EBPα protein levels were also significantly increased compared to controls. Notably, overexpressing C/EBPα did not affect hepatic C/EBPβ protein levels (Figure 3.12A). We also observed increased gene expression of *Hsd11b1* (Figure 3.12B), suggesting that C/EBPα overexpression led to increased hepatic C/EBPα activity at the *Hsd11b1* promoter. However, C/EBPα overexpression did not improve the circulating albumin level in AktDKO animals (Figure 3.12C). To our surprise, albumin gene expression was further decreased in animals overexpressing C/EBPα (Figure 3.12D).

To understand this surprising result, we first assessed the effect of C/EBPα overexpression on restoring the reduced C/EBPα binding to DNA. We found that the overexpression failed to increase C/EBPα occupancy at the promoters of *Alb*, *Cebpa*, and *Hsd11b1* in AktDKO livers (Figure 3.13A). This is likely the reason why C/EBPα overexpression failed to rescue the reduced albumin expression and production in AktDKO livers. In addition, C/EBPβ occupancy at these sites was significantly decreased as a result of the C/EBPα overexpression (Figure 3.13B). Since C/EBPβ is also a transcription activator of albumin, this might explain the further decrease in hepatic albumin mRNA levels.

Discussion

The *Alb* promoter does not contain a Foxo1 binding site, suggesting that it is unlikely for Foxo1 to repress albumin expression directly. In this chapter, we investigated possible mechanisms by which Foxo1 represses albumin expression indirectly.

Shp and Id3 are putative transcription repressors downstream of Foxo1 that might play a role in the regulation of albumin expression. Specifically, Shp has been described to directly interact with C/EBPα and to decrease its transcriptional activity (Park et al., 2007). Since C/EBPα activates albumin expression, we hypothesized that the induction of Shp by Foxo1 could potentially mediate the downregulation of albumin gene expression. Id3 exhibits reciprocal expressions both in space and in time with albumin during chick liver development, consistent with it being an inducible repressor for albumin expression (Nakayama et al., 2006). We found that the gene expression of Shp and Id3 were elevated in Akt-null livers, where Foxo1 activity was constitutively high, and concomitant deletion of *Foxo1* reversed this induction (Figure 3.1A and B). The reciprocal relationship between albumin expression and the expression of Shp and Id3 is consistent with the model where Shp and Id3 repress albumin transcription downstream of Foxo1. However, the expression patterns of *Shp* and *Id3* in control animals were inconsistent with the model where Foxo1 induces these transcription repressors during the fasting state (Figure 3.1 A-C), suggesting that Foxo1 might not be a physiologic regulator of these genes. In addition, we did not observe the same relationship in the IRKO mice (Figure 3.1C). The discrepancy between the IRKO and AktDKO models also suggests

that regulation of Shp and Id3 is more complex and possibly depends on regulators other than Foxo1. Therefore, repression mediated by Shp and Id3 is unlikely to be the unifying mechanism of how Foxo1 represses albumin gene expression.

Since a number of studies have demonstrated that Foxo1 interacts with a variety of transcription factors to modulate their activity (Christian, 2002; Deng et al., 2012; Dowell, 2003; Fan et al., 2009; Ramaswamy et al., 2002; Van der Vos and Coffer, 2008), we then turned our attention to transcription activators known to regulate albumin and assessed whether this is the mechanism by which Foxo1 exerts its repressive effect on albumin gene expression. Interestingly, under certain conditions, hepatic expression of *Cebpa* inversely correlated with Foxo1 activity and consistent with albumin expression. Specifically, in IRKO and AktDKO livers where Foxo1 was constitutively active, *Cebpa* exhibited decreased expression that was completely reversed by concomitant deletion of *Foxo1* especially in the fasted state. Since C/EBPα regulates its own transcription, this observation suggests that C/EBPα activity exhibits a reciprocal relationship with Foxo1 activity in the liver, consistent with the model in which Foxo1 inhibits the activity of C/EBPα to mediate the repression of albumin expression. In the fed state, however, inactivation of Foxo1 was not sufficient to restore the reduced *Cebpa* expression in Akt-null livers, suggesting that there are other nutrient-dependent, Foxo1-independent pathways that regulate *Cebpa* expression.

To test the reciprocal relationship between C/EBPα and Foxo1, we measured the expression of *Hsd11b1*, a direct target gene of C/EBPα that is

involved in glucocorticoid synthesis in liver (Inoue et al., 2004; Williams et al., 2000). Consistent with the observed expression patterns of albumin and *Cebpa*, *Hsd11b1* also exhibited significantly reduced expression in AktDKO livers that was reversed when *Foxo1* was concomitantly deleted (Figure 3.7A), providing another piece of supporting evidence that C/EBPα activity was decreased when Foxo1 activity was constitutively high, and vice versa. We then compiled a list of genes that are most likely the functional C/EBPα targets by examining the overlap between genes whose promoters are bound by C/EBPα (C/EBPα ChIP, (Jakobsen et al.)) and genes with differential expression when *Cebpa* is deleted (*Cebpa* knockout microarray, (Pedersen et al., 2007)). We found that almost all C/EBPα target genes exhibited differential expression that was dependent on Foxo1 activity (Figure 3.7C), further validating the model that Foxo1 interferes with C/EBPα activity.

The decrease in C/EBPα activity in IRKO and AktDKO livers was not due to a loss of C/EBPα protein, as there was no detectable change in C/EBPα protein in IRKO and AktDKO livers, and concomitant deletion of *Foxo1* in these livers exhibited no effect on C/EBPα protein levels (Figure 3.5A and Figure 3.6A). This suggests that decreased C/EBPα activity in IRKO and AktDKO livers was due to a decrease in the inherent transcription activating activity. Interestingly, C/EBPβ protein was regulated differently as C/EBPα and was dependent on Foxo1 activity, as we observed a mild increase in C/EBPβ protein in both IRKO and AktDKO livers compared to control, and concomitant deletion of *Foxo1* completely reversed this increase (Figure 3.5B and Figure 3.6B). These

observations highlight differential regulations of C/EBPα and C/EBPβ downstream of Foxo1: C/EBPα seems to be regulated at the level of transcription, while C/EBPβ is regulated post-transcriptionally at the protein level either by translation or degradation.

Recent studies have revealed that Foxo1 can modulate gene expression independent of DNA-binding by associating with a variety of transcription factors (Christian, 2002; Deng et al., 2012; Dowell, 2003; Fan et al., 2009; Hirota et al., 2008; Ramaswamy et al., 2002; Van der Vos and Coffer, 2008). For instance, identified from a yeast two-hybrid screen, Foxo1 physically interacts with peroxisome proliferator-activated receptor γ (PPARγ) and disrupts PPARγ's DNA-binding to antagonize its activity (Dowell, 2003). Recently, Deng et al. found that Foxo1 inhibits SREBP-1c transcription by disrupting the assembly of the transcriptional complex at the *Srebp1c* promoter (Deng et al., 2012). In addition, it has been shown that Foxo1 physically interacts with C/EBPα in adipocytes and neonatal liver (Qiao and Shao, 2006; Sekine et al., 2007). Collectively, these studies led us to speculate that Foxo1 may antagonize C/EBPα activity by directly interacting with C/EBPα and interfering with its DNA binding.

To assess C/EBPα binding to the albumin promoter, we performed chromatin immunoprecipitation in control, AktDKO, and FoxoTKO livers using an antibody targeted specifically at C/EBPα. We found that C/EBPα occupancy at the albumin promoter was decreased in AktDKO livers compared to control, especially at the proximal promoter region (approximately 2.5kb upstream from

the transcription start site). Moreover, concomitant deletion of *Foxo1* restored the decreased occupancy to control level (Figure 3.8B). This observation is consistent with our hypothesis that constitutively active Foxo1 may interfere with C/EBPα's binding to DNA and thus lead to repression of the albumin gene. We also observed a decrease in C/EBPβ occupancy at the albumin promoter (Figure 3.8C). It is possible that C/EBPβ binding to the albumin promoter was decreased as a result of disrupted C/EBPα binding, since these factors often bind to DNA as a heterodimer. Interestingly, C/EBPα and C/EBPβ occupancy did not exhibit the same pattern at the distal promoter region (site 4, approximately 30kb upstream from the transcription start site), suggesting that at this site, C/EBPα and C/EBPβ interacted with the promoter as homodimers rather than a heterodimer: DNA-binding of C/EBPα homodimer at this site was disrupted by Foxo1, whereas that of C/EBPβ homodimer was unaffected.

We also examined the occupancy of C/EBPα and C/EBPβ at the promoters of *Cebpa* and *Hsd11b1* in control, AktDKO, and FoxoTKO livers. Constitutively active Foxo1 in AktDKO livers was correlated to a slight, yet statistically insignificant, decrease in C/EBPα DNA binding to the promoters of these genes. Nevertheless, concomitant deletion of *Foxo1* still restored the slight decrease to control levels, suggesting that C/EBPα occupancy was dependent on Foxo1 (Figure 3.10B and Figure 3.11B). On the contrary, C/EBPβ binding to these promoters was unaffected by Foxo1 activity level (Figure 3.10C and Figure 3.11C). Such disconnect between C/EBPα and C/EBPβ occupancy could be explained by the possibility that C/EBPα and C/EBPβ interacted with these sites

as homodimers exclusively, and Foxo1 only interfered with C/EBPα binding. Since gene expression for both *Cebpa* and *Hsd11b1* was significantly decreased in AktDKO livers without a significant decrease in C/EBPα occupancy at the promoter, it is likely that other factors contributed to the repression of these genes as well.

To directly investigate the role of C/EBPα and test whether reduced C/EBPα occupancy is a major contributing factor for decreased albumin gene expression, we overexpressed C/EBPα in AktDKO livers in an attempt to rescue the decreased C/EBPα occupancy to see if that is sufficient to restore albumin production. Using an adeno-associated virus, we achieved a 4-fold overexpression in *Cebpa* mRNA, and C/EBPα protein levels were significantly increased compared to controls (Figure 3.12A and B). *Hsd11b1* gene expression also significantly increased, suggesting that C/EBPα activity was elevated in these livers as a result of the overexpression (Figure 3.12B). Importantly, C/EBPα overexpression exhibited no effect on C/EBPβ gene expression and protein levels in AktDKO livers (Figure 3.12A and B). C/EBPα overexpression in AktDKO livers did not improve total serum protein concentration in these animals (Figure 3.12C). Opposite to what we expected, C/EBPα overexpression actually exacerbated the already decreased albumin gene expression in AktDKO livers (Figure 3.12D). Chromatin immunoprecipitation against C/EBPα revealed that despite of the increased protein levels, C/EBPα occupancy at the promoters of albumin, *Cebpa*, and *Hsd11b1* did not increase as a result of the overexpression (Figure 3.13A). Thus, it remains inconclusive whether decreased C/EBPα

binding to the albumin promoter is the major contributing factor for decreased albumin expression in AktDKO livers. It is also interesting to note that even though C/EBPα occupancy at the *Hsd11b1* promoter was not rescued in *Cebpa*-overexpressing livers, *Hsd11b1* expression was nonetheless elevated, suggesting that increased DNA-binding might not be the only mechanism C/EBPα has to activate gene expression. Interestingly, C/EBPβ binding to the promoters of albumin, *Cebpa*, and *Hsd11b1* was significantly decreased as a result of *Cebpa* overexpression (Figure 3.13B), possibly due to the distorted stoichiometric ratio of C/EBPα and C/EBPβ in these livers. Since C/EBPβ is also a transcription activator of albumin expression, loss of C/EBPβ at the promoter of albumin could potentially contribute to the additional decrease in albumin expression in *Cebpa*-overexpressing AktDKO livers.

It is not clear why overexpressing *Cebpa* in AktDKO livers failed to restore the decreased C/EBPα occupancy at the albumin promoter. One possibility is that C/EBPα binding sites become inaccessible when *Akt* is deleted in the liver. This could be caused by altered chromatin structure. A recent study by Lee et al. demonstrates that Akt activity is correlated with histone acetylation level, which is known to regulate chromatin structure (Lee et al., 2014). In Akt-null liver, histone acetylation level is likely low and the chromatin is likely more compact, which could contribute to reduced binding of C/EBPα. Alternatively, it is possible that C/EBPα binding to the albumin promoter requires a cofactor that becomes downregulated in Akt-null liver. The third possibility is that C/EBPα binding sites

could be occupied by another transcription factor that is upregulated in Akt-null liver. The nature of these factors, however, is currently elusive.

As previous discussed, site 2 of the albumin promoter was most likely occupied by C/EBP α/β heterodimers, since the DNA binding of both factors was disrupted concomitantly (Figure 3.8B and C). Instead of restoring the decreased occupancy of C/EBP α/β heterodimers at these sites, *Cebpa* overexpression increased the relative C/EBPα to C/EBPβ ratio in the cell and possibly led to a displacement of C/EBPβ, as suggested by the decrease in C/EBPβ occupancy (Figure 3.13A and B). As a result, this site likely became occupied by C/EBP α/α homodimers. Similarly, site 4 of the albumin promoter was possibly occupied by C/EBP α/α and C/EBP β/β homodimers, as only the occupancy of C/EBPα was dependent on Foxo1 activity but not that of C/EBPβ (Figure 3.8B and C). We speculate that *Cebpa* overexpression displaced C/EBPβ, resulting in a loss of C/EBP β/β homodimers at this site.

Sequential ChIP assay could be useful here to directly test the relative protein dimeric states at these sites. Specifically, C/EBPα ChIP samples would be re-ChIPed by the C/EBPβ antibody. By comparing the relative enrichment from first ChIP with that from the second ChIP, one may gain insights on the nature of the dimer occupying that particular site: A site bound by C/EBP α/β heterodimer would exhibit preserved enrichment after re-ChIP with C/EBPβ antibody, whereas a site bound by C/EBP α/α homodimer would lose enrichment after C/EBPβ re-ChIP. The reverse order - C/EBPβ ChIP followed by C/EBPα ChIP - could be used to test occupancy by C/EBP β/β homodimers. This

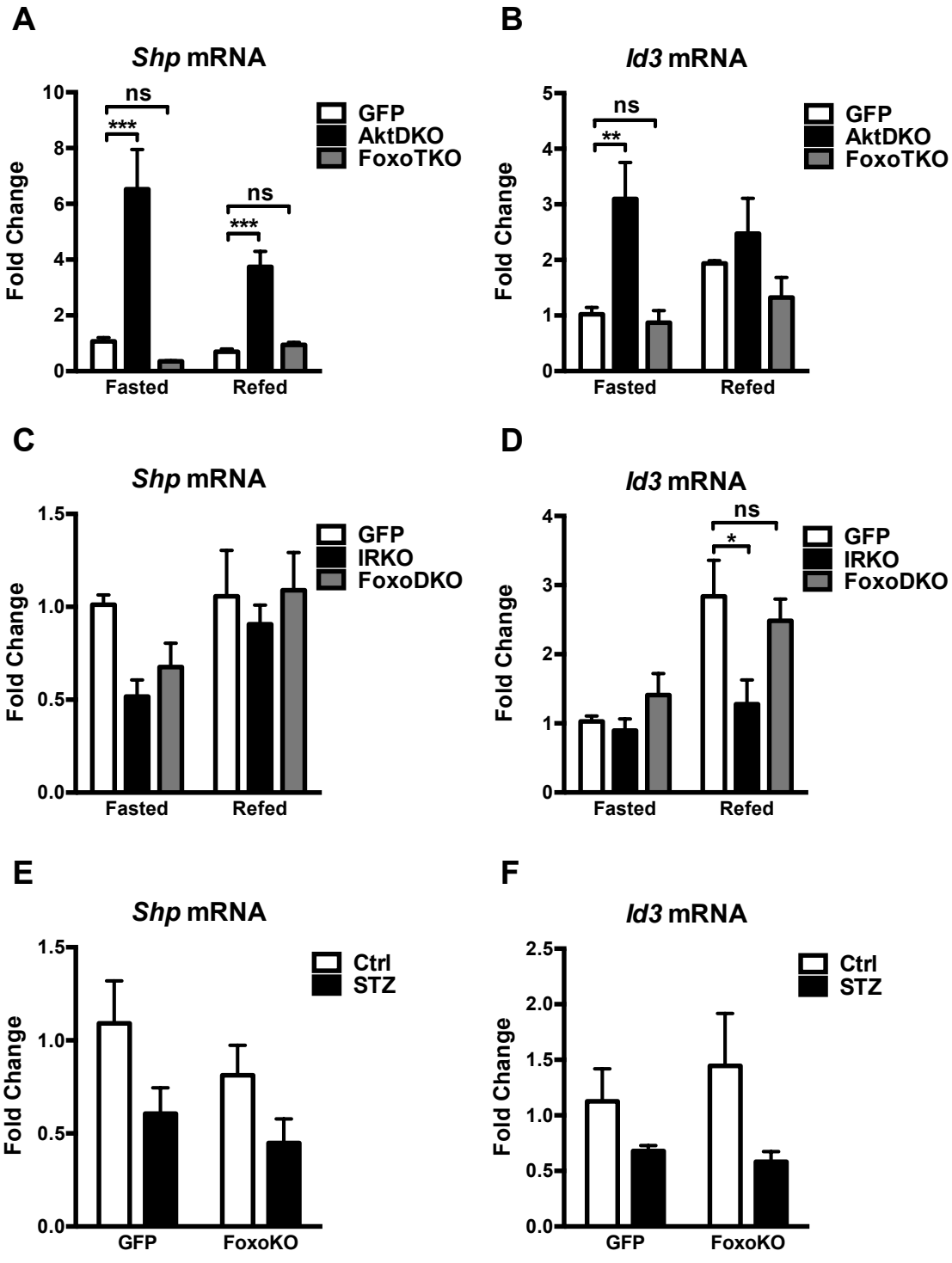
experimental system would also be useful to assess the effect of over-expressing *Cebpa* on the C/EBP dimeric states.

We did not observe similar effects in the STZ-induced Type 1 diabetic model. C/EBPα binding to the albumin promoter was only reduced at the proximal site, but not at the distal site as a result of diabetes (Figure 3.9A). Interestingly, STZ treatment led to a significant increase in C/EBPβ occupancy, and deletion of *Foxo1* completely reversed this increase, suggesting that the enhanced C/EBPβ binding to the albumin promoter was dependent on Foxo1 activity (Figure 3.9B). Similar trend was also observed at the *Cebpa* and *Hsd11b1* promoters, where STZ treatment did not affect C/EBPα binding, but constitutive activation of Foxo1 was correlated with enhanced C/EBPβ binding at these sites (Figure 3.10D and E, Figure 3.11D and E). C/EBPβ exits as two isoforms: one activating and one inhibitory (Nerlov, 2007; 2008). The C/EBPβ antibody we used in ChIP experiments does not differentiate the two isoforms. Therefore, it is possible that the occupancy of the inhibitory C/EBPβ isoform was increased in STZ-treated livers, thus causing a decrease in albumin gene expression. The specific C/EBPβ isoform recruited to the albumin promoter in this model requires further characterization.

The discrepancy between the STZ-induced Type 1 diabetes model and the liver-specific genetic knockout model may be explained by either the genetic strain differences of the animals used in this study, or the drastic physiological differences between the two animal models. Although liver-specific *Ir* knockout and liver-specific *Akt1*/*Akt2* double-knockout animals develop peripheral insulin

resistance (Lu et al., 2012), the metabolic defect originated from the liver. On the other hand, β-cell death following the STZ treatment leads to a systemic defect that affects all insulin-responsive organs, including liver, adipose, muscle, and brain. As a result, the severity of the metabolic defects is much higher in the STZ-induced diabetes model, as indicated by their outrageously high blood glucose level as well as the significant loss of body mass. It is thus difficult to de-convolute the liver-specific mechanism that regulates albumin expression using the STZ-induced diabetes model.

Figures


A
Shp mRNA
Fold Change
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GFP
AktDKO
FoxoTKO
Fasted
Refed
C
Shp mRNA
Fold Change
GFP
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FoxoDKO
Fasted
Refed
D
Id3 mRNA
Fold Change
ns
*
GFP
IRKO
FoxoDKO
Fasted
Refed
E
Shp mRNA
Fold Change
Ctrl
STZ
GFP
FoxoKO
F
Id3 mRNA
Fold Change
Ctrl
STZ
GFP
FoxoKO

Figure 3.1: Shp and Id3 are unlikely to be the transcriptional repressors downstream of Foxo1 to repress albumin expression.

A,B. Hepatic gene expression level of small heterodimer partner (Shp, A) and inhibitor of DNA binding 3 (Id3, B) in GFP control (GFP), liver-specific *Akt1*/*Akt2* double-knockout (AktDKO), and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) animals that had been either fasted overnight or fasted overnight and refed for 4 hours. n = 3-4; ns, not significant, **p<0.01 vs. GFP, and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

C,D. Hepatic gene expression level of Shp (C) and Id3 (D) in GFP, liver-specific *Ir* knockout (IRKO), and liver-specific *Ir*/*Foxo1* double-knockout (FoxoDKO) animals that had been either fasted overnight or fasted overnight and refed for 4 hours. n = 5-7; ns, not significant, *p<0.0.5 vs. GFP by two-way ANOVA using Sidak post-test.

E,F. Hepatic gene expression level of Shp (E) and Id3 (F) in GFP and liver-specific *Foxo1* knockout (FoxoKO) animals 9 days post an intra-peritoneal injection of either buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight. n = 5-7.

All values are expressed as mean ± SEM.

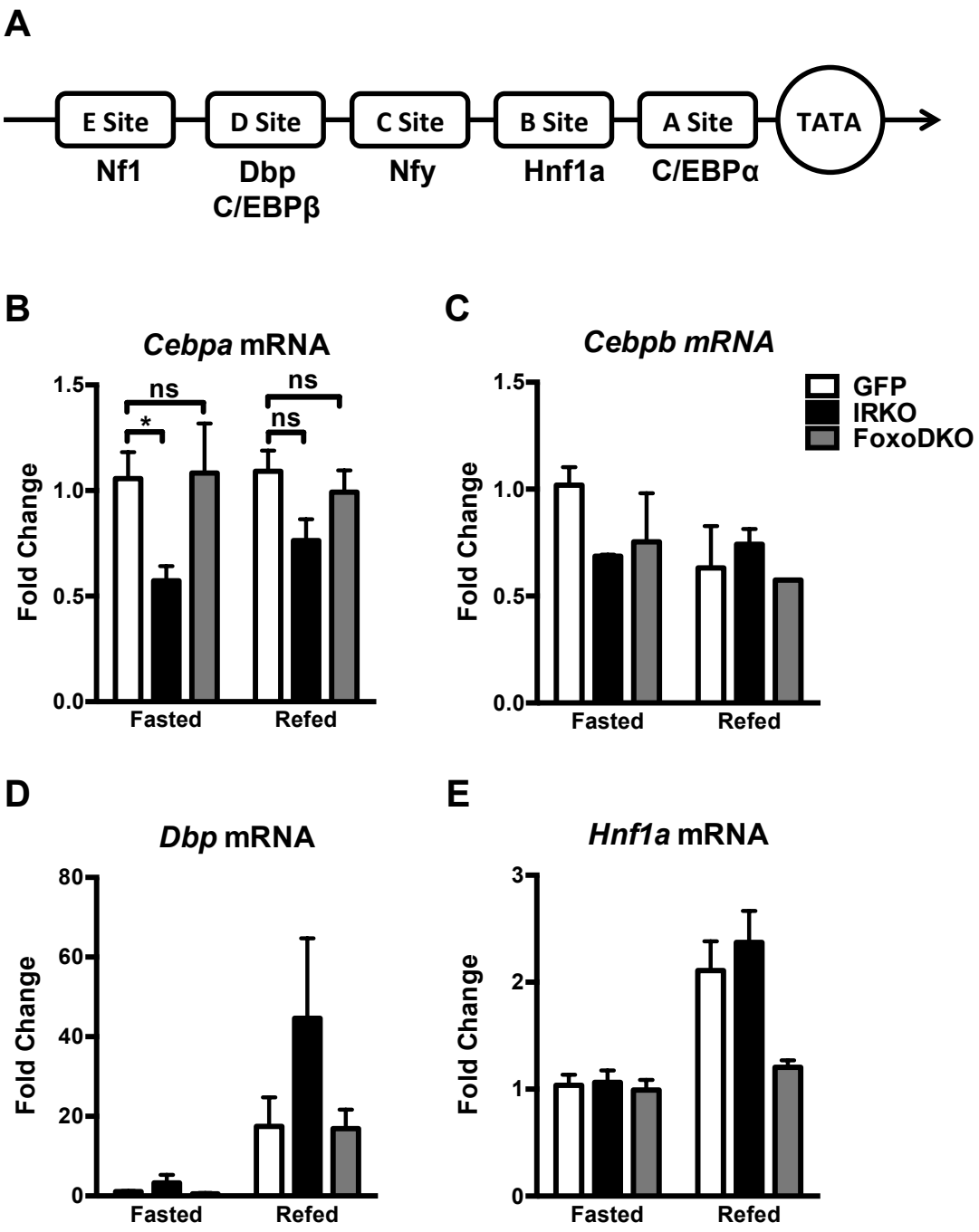
A
E Site
D Site
C Site
B Site
A Site
TATA
Nf1
Dbp
C/EBPβ
Nfy
Hnf1a
C/EBPα
B
Cebpa mRNA
Fold Change
1.5
1.0
0.5
0.0
ns
*
ns
ns
Fasted
Refed
C
Cebpb mRNA
GFP
IRKO
FoxoDKO
D
Dbp mRNA
80
60
40
20
0
E
Hnf1a mRNA
3
2
1
0

Figure 3.2: Hepatic expression of transcription factors known to regulate albumin expression in liver-specific *Ir* knockout (IRKO) and liver-specific *Ir*/*Foxo1* double-knockout (FoxoDKO) mice.

A. Diagram of *Alb* promoter showing transcription factors that activate albumin expression and their respective binding sites. Cebpa/b, CCAAT/enhancer binding protein a/b; Hnf1a, hepatic nuclear factor 1a; Nfy, nuclear factor Y; Dbp, D box binding protein; Nf1, neurofibromatosis factor 1.

B-E. Hepatic gene expression of *Cebpa* (B), *Cebpb* (C), *Dbp* (D), and *Hnf1a* (E) in GFP control (GFP), IRKO, and FoxoDKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

All values are expressed as mean ± SEM. n = 5-9; ns, not significant; *p<0.05 vs. GFP by two-way ANOVA using Sidak post-test.

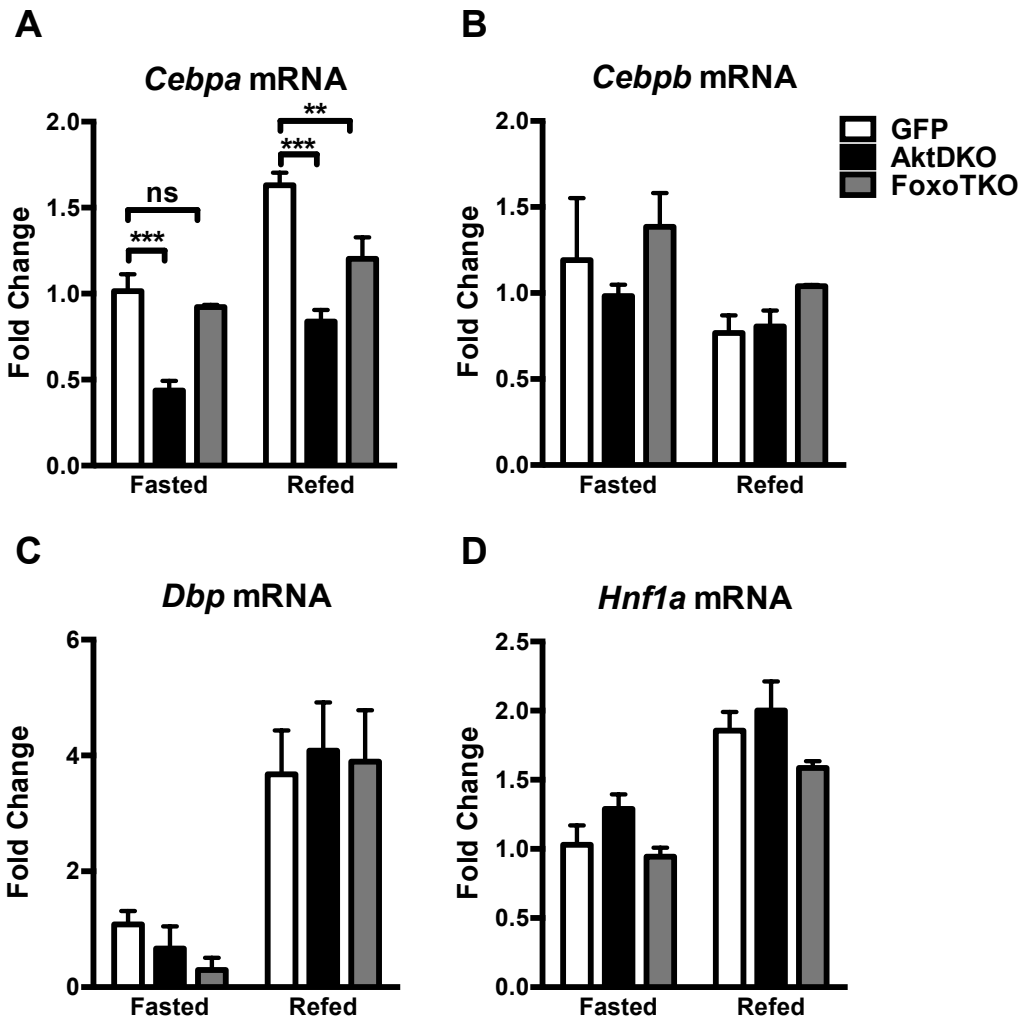

Figure 3.3: Hepatic expression of transcription factors known to regulate albumin expression in liver-specific *Akt1*/*Akt2* double-knockout (AktDKO) and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) mice.

Hepatic gene expression of CCAAT/enhancer binding protein a (Cebpa, A), Cebpb (B), D box binding protein (Dbp, C), and hepatic nuclear factor 1a (Hnf1a, D) in GFP control (GFP), AktDKO, and FoxoTKO animals that had been either fasted overnight or fasted overnight and refed for 4 hours.

All values are expressed as mean ± SEM. n = 3-4; ns, not significant, **p<0.01 vs. GFP, and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

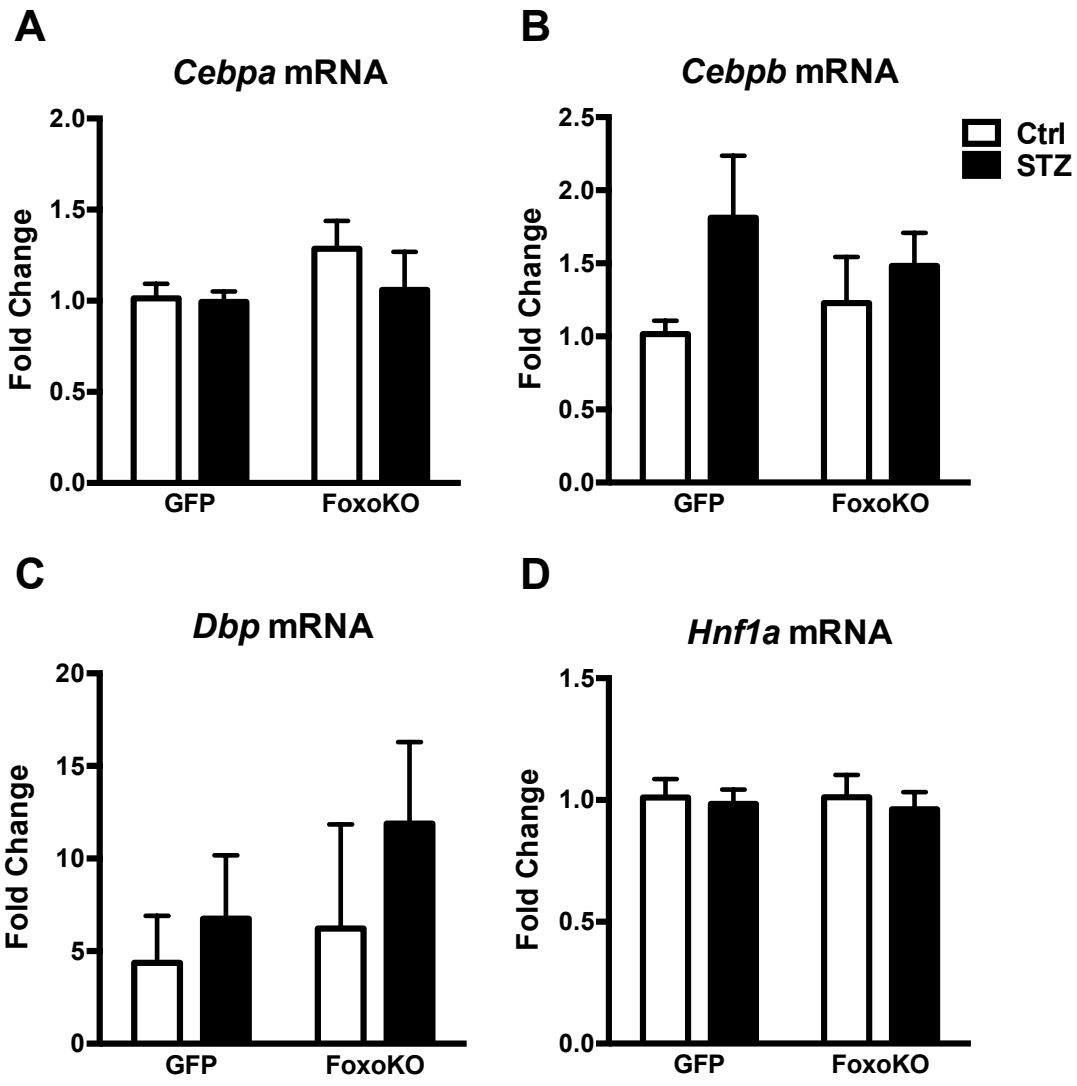

Figure 3.4: Hepatic expression of transcription factors known to regulate albumin expression in Type 1 diabetic livers.

Hepatic gene expression of CCAAT/enhancer binding protein a (Cebpa, A), Cebpb (B), D box binding protein (Dbp, C), and hepatic nuclear factor 1a (Hnf1a, D) in GFP control (GFP) and liver-specific *Foxo1* knockout (FoxoKO) animals 9

days post an intra-peritoneal injection of either buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight.

All values are expressed as mean ± SEM.

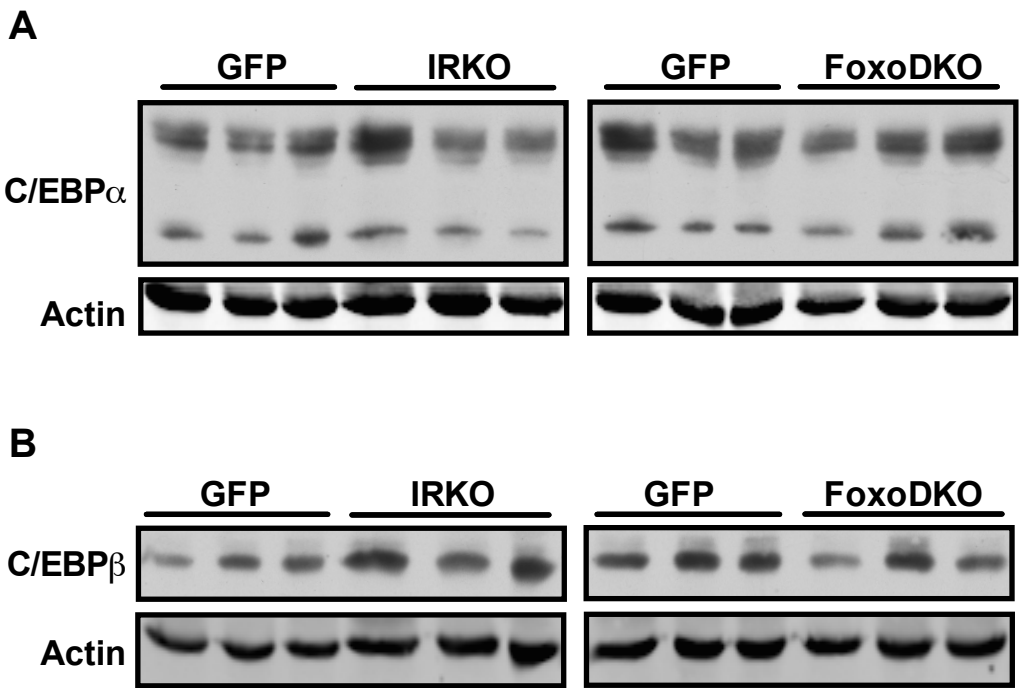

Figure 3.5: Hepatic C/EBPα and C/EBPβ protein levels in liver-specific *Ir* knockout (IRKO) and liver-specific *Ir*/*Foxo1* double-knockout (FoxoDKO) mice.

Western blots for C/EBPα (A), C/EBPβ (B), and actin in liver homogenates of GFP control (GFP), IRKO, and FoxoDKO animals.

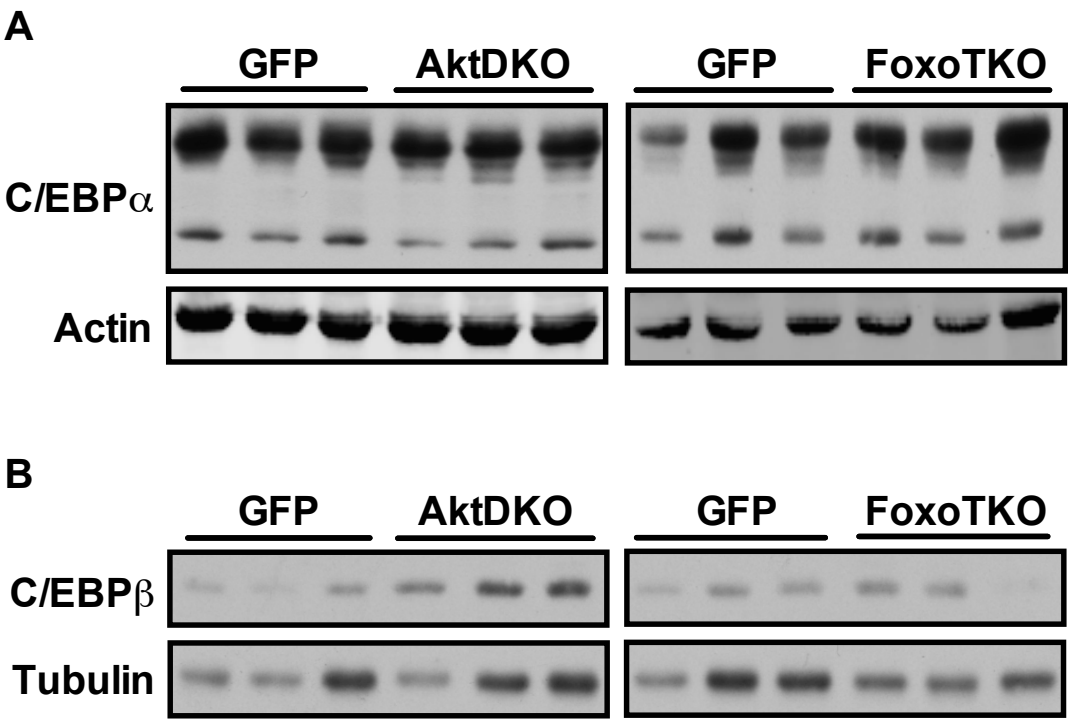

Figure 3.6: Hepatic C/EBPα and C/EBPβ protein levels in liver-specific *Akt1*/*Akt2* double-knockout (AktDKO) and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) mice.

Western blots for C/EBPα (A), C/EBPβ (B), and actin in liver homogenates of GFP control (GFP), AktDKO, and FoxoTKO animals.

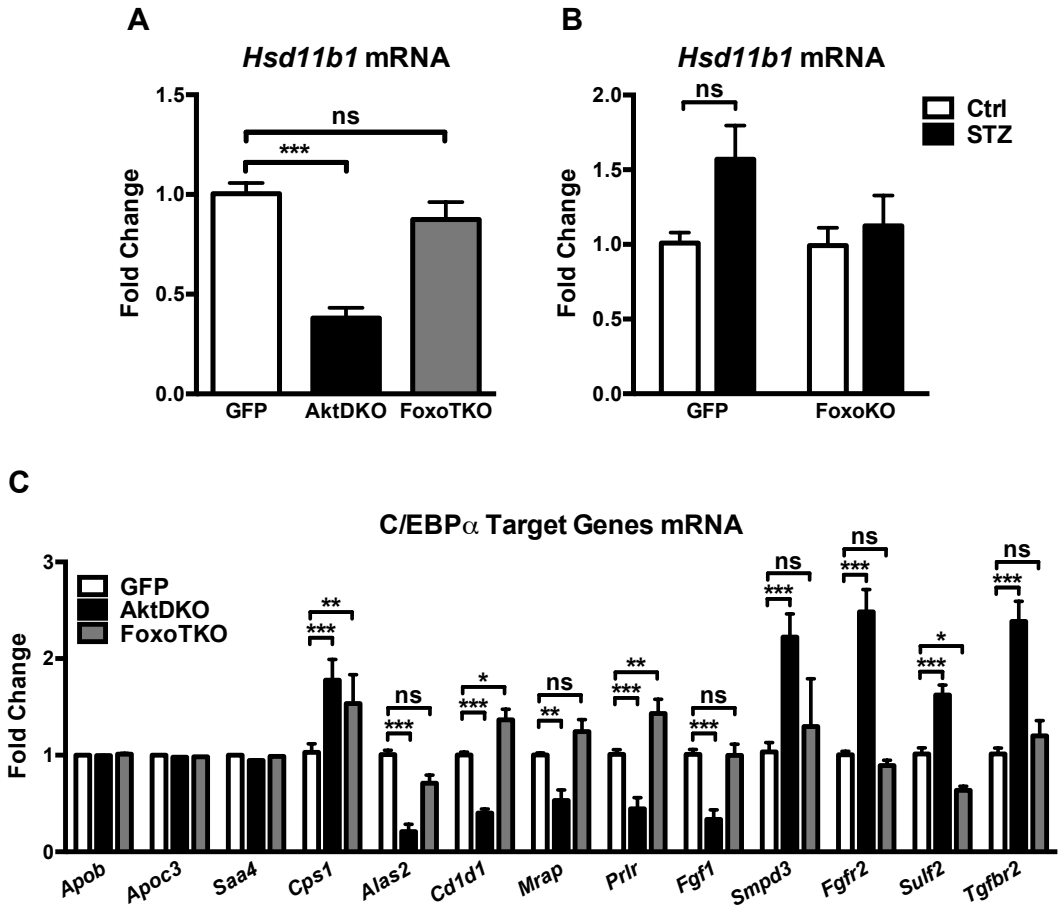

Figure 3.7: C/EBPα target genes exhibit differential expression in *Akt*-null livers that is restored by additional deletion of *Foxo1.*

A. Hepatic *Hsd11b1* mRNA level in GFP control (GFP), liver-specific *Akt1*/*Akt2* double knockout (AktDKO), and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) animals. n = 3-4; ns, not significant, and ***p<0.001 vs. GFP by one-way ANOVA using Sidak post-test.

B. Hepatic *Hsd11b1* mRNA in GFP and liver-specific *Foxo1* knockout (FoxoKO) animals 9 days post an intra-peritoneal injection of either buffer (Ctrl) or

streptozotocin (STZ) at 200mg per kg body weight. n = 5-7; ns, not significant by two-way ANOVA using Sidak post-test.

C. Hepatic mRNA levels of other putative target genes of C/EBPα in GFP, AktDKO, and FoxoTKO animals. *Apob*, apolipoprotein B; *Apoc3*, apolipoprotein C-III; *Saa4*, serum amyloid A4; *Cps1*, carbamoyl-phosphate synthetase 1; *Alas2*, aminolevulinic acid synthase 2; *Cd1d1*, CD1d1 antigen; *Mrap*, melanocortin 2 receptor accessory protein; *Prlr*, prolactin receptor; *Fgf1*, fibroblast growth factor 1; *Smpd3*, sphingomyelin phosphodiesterase 3; *Fgfr2*, fibroblast growth factor receptor 2; *Sulf2*, sulfatase 2; *Tgfbr2*, transforming growth factor, beta receptor II. n = 3-4; ns, not significant, *p<0.05 vs. GFP, **p<0.01 vs. GFP, and ***p<0.001 vs. GFP by two-way ANOVA using Sidak post-test.

All values are expressed as mean ± SEM.

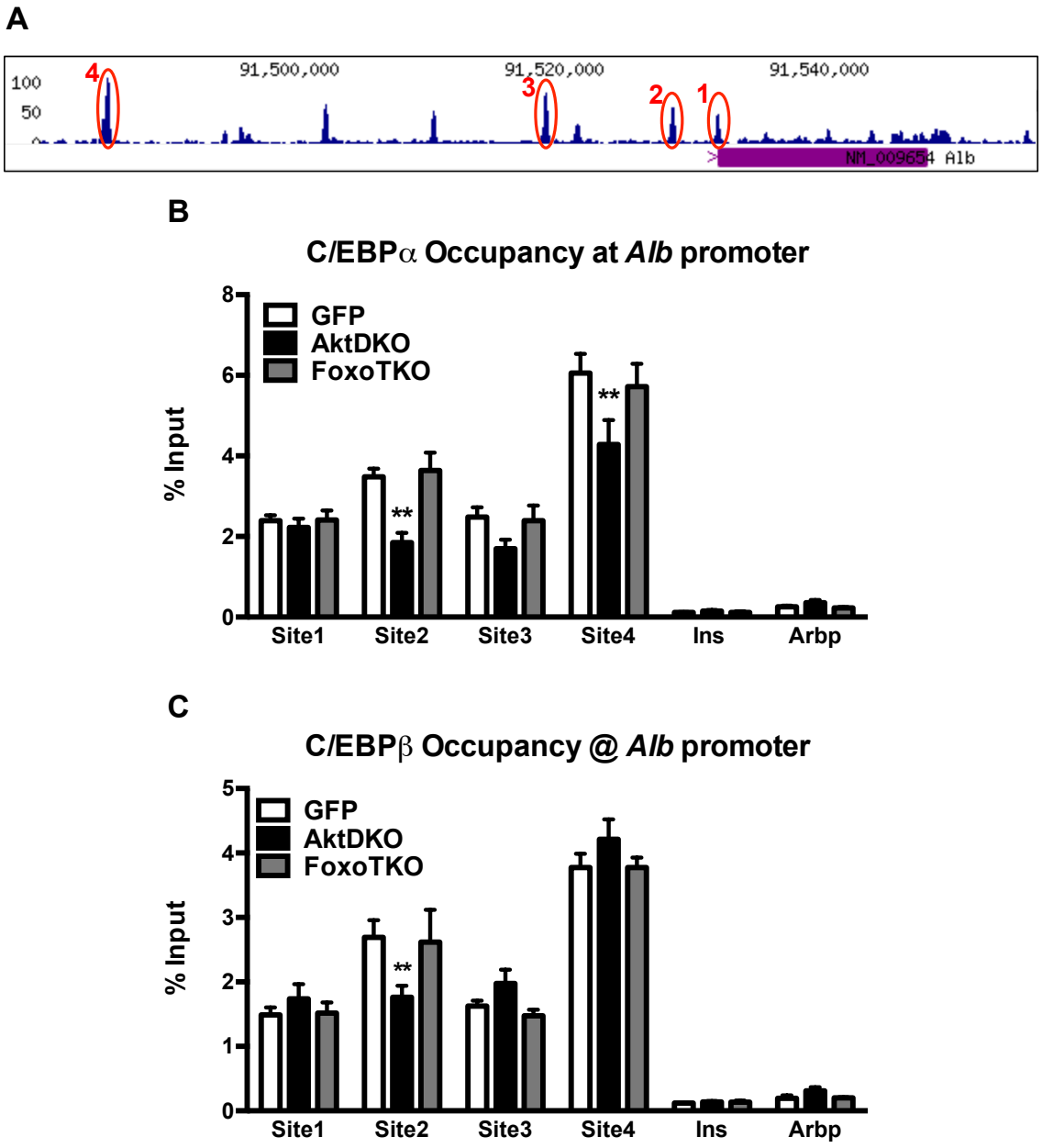

Figure 3.8: C/EBPα and C/EBPβ binding to the albumin promoter negatively correlates with hepatic Foxo1 activity.

A. ChIP-seq profile for C/EBPβ at the albumin promoter. C/EBPβ binding sites selected for analyzing C/EBPα and C/EBPβ enrichment are indicated.

B, C. Hepatic enrichment of C/EBPα (A) and C/EBPβ (B) at indicated sites of the albumin promoter in GFP control (GFP), liver-specific *Akt1*/*Akt2* double-knockout (AktDKO), and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) animals. Ins serves as a negative control site not bound by C/EBPα.

All values are expressed as mean ± SEM. n = 3-4; **p<0.01 vs. GFP by one-way ANOVA using Sidak post-test.

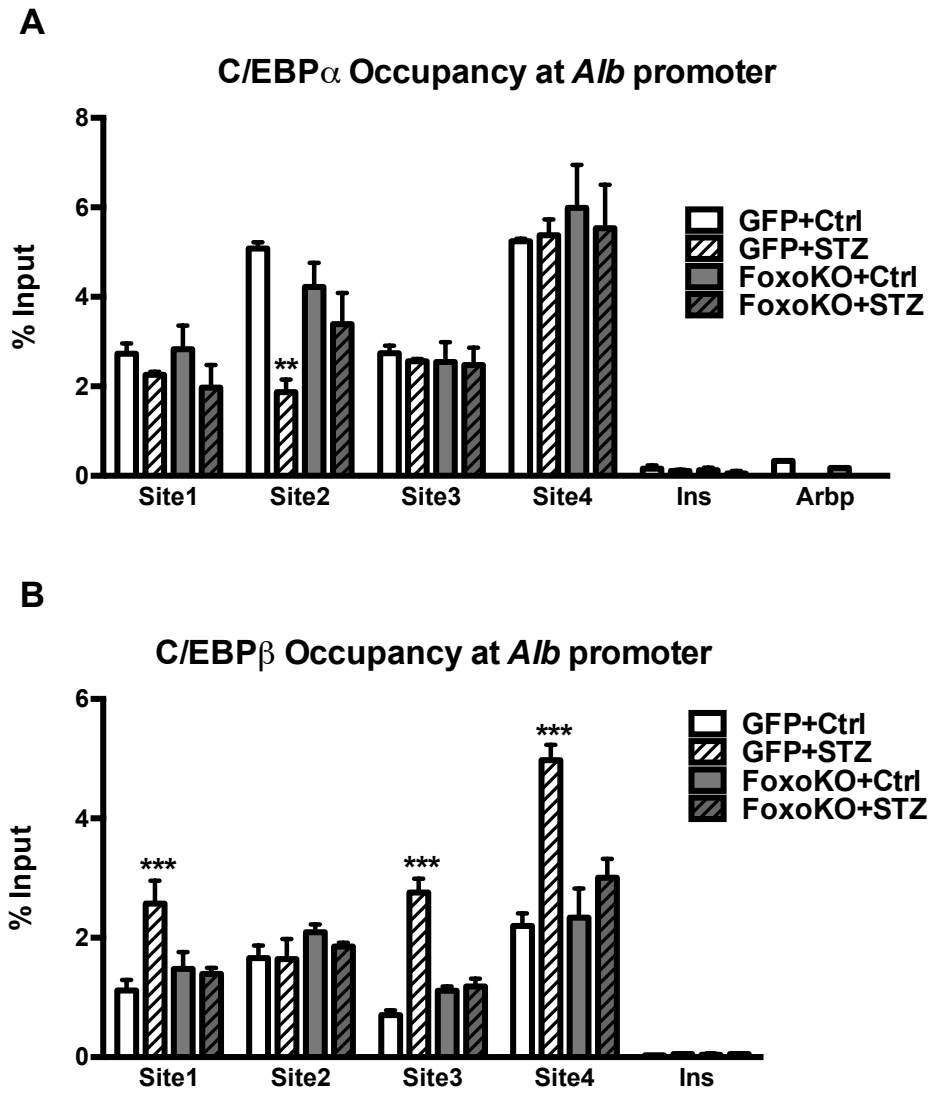

Figure 3.9: C/EBPα and C/EBPβ binding to the albumin promoter in diabetic liver.

Hepatic enrichment of C/EBPα (A) and C/EBPβ (B) at indicated sites (See Figure 3.10A) of the albumin promoter in GFP control (GFP) and liver-specific *Foxo1* knockout (FoxoKO) animals 9 days post an intra-peritoneal injection of either

buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight. Ins and Arbp serve as negative control sites not bound by C/EBPα.

All values are expressed as mean ± SEM. n = 3; **p<0.01 vs. GFP+Ctrl, and ***p<0.001 vs. GFP+Ctrl by two-way ANOVA using Sidak post-test.

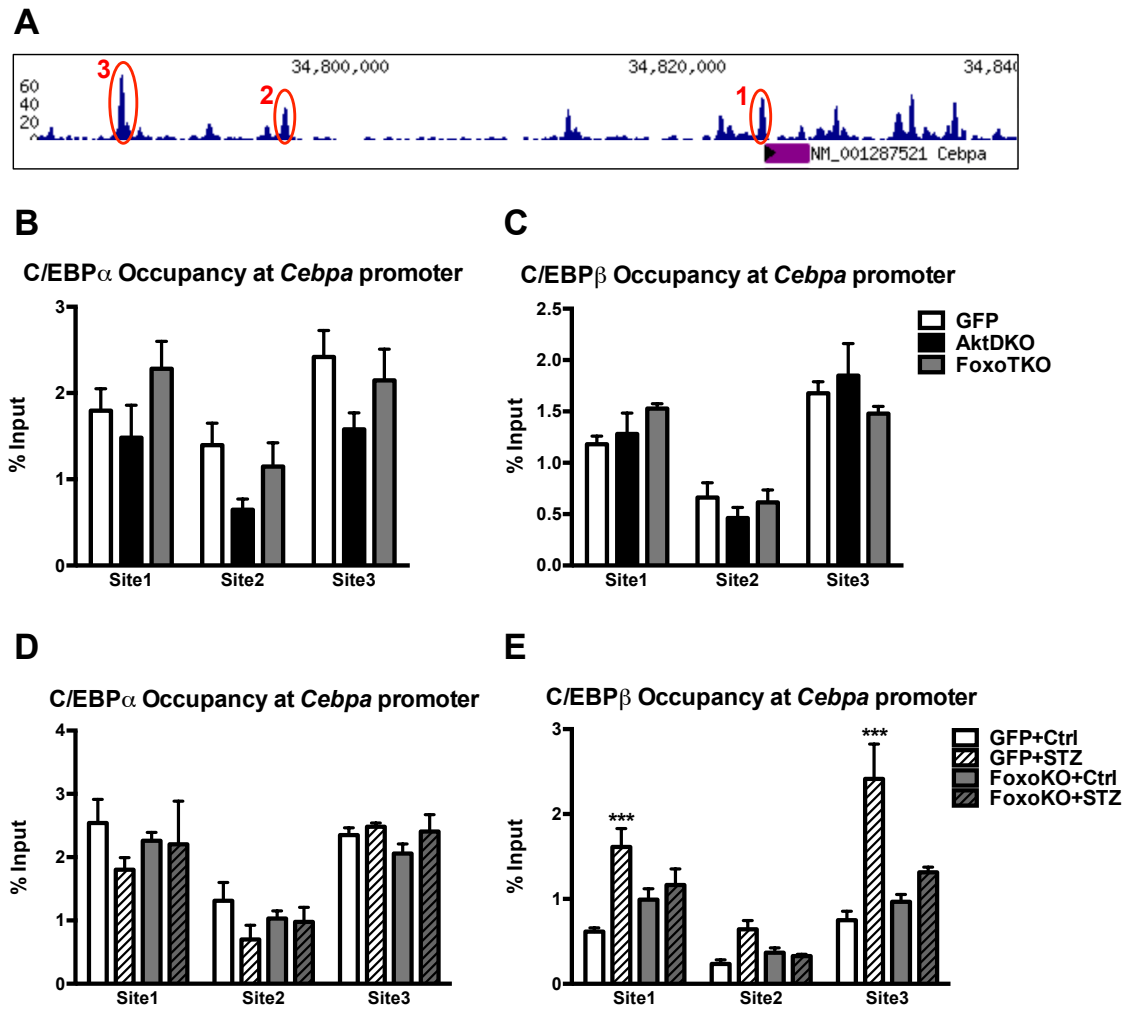

Figure 3.10: C/EBPα and C/EBPβ binding to the *Cebpa* promoter.

A. ChIP-seq profile for C/EBPβ at the *Cebpa* promoter. C/EBPβ binding sites selected for analyzing C/EBPα and C/EBPβ enrichment are indicated.

B,C. Hepatic enrichment of C/EBPα (B) and C/EBPβ (C) at indicated sites of the *Cebpa* promoter in GFP control (GFP), liver-specific *Akt1*/*Akt2* double-knockout (AktDKO), and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout (FoxoTKO) animals.

D,E. Hepatic enrichment of C/EBPα (C) and C/EBPβ (D) at indicated sites of the *Cebpa* promoter in GFP and liver-specific *Foxo1* knockout (FoxoKO) animals 9 days post an intra-peritoneal injection of either buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight.

All values are expressed as mean ± SEM. n = 3-4; ***p<0.001 vs. GFP+Ctrl by two-way ANOVA using Sidak post-test.

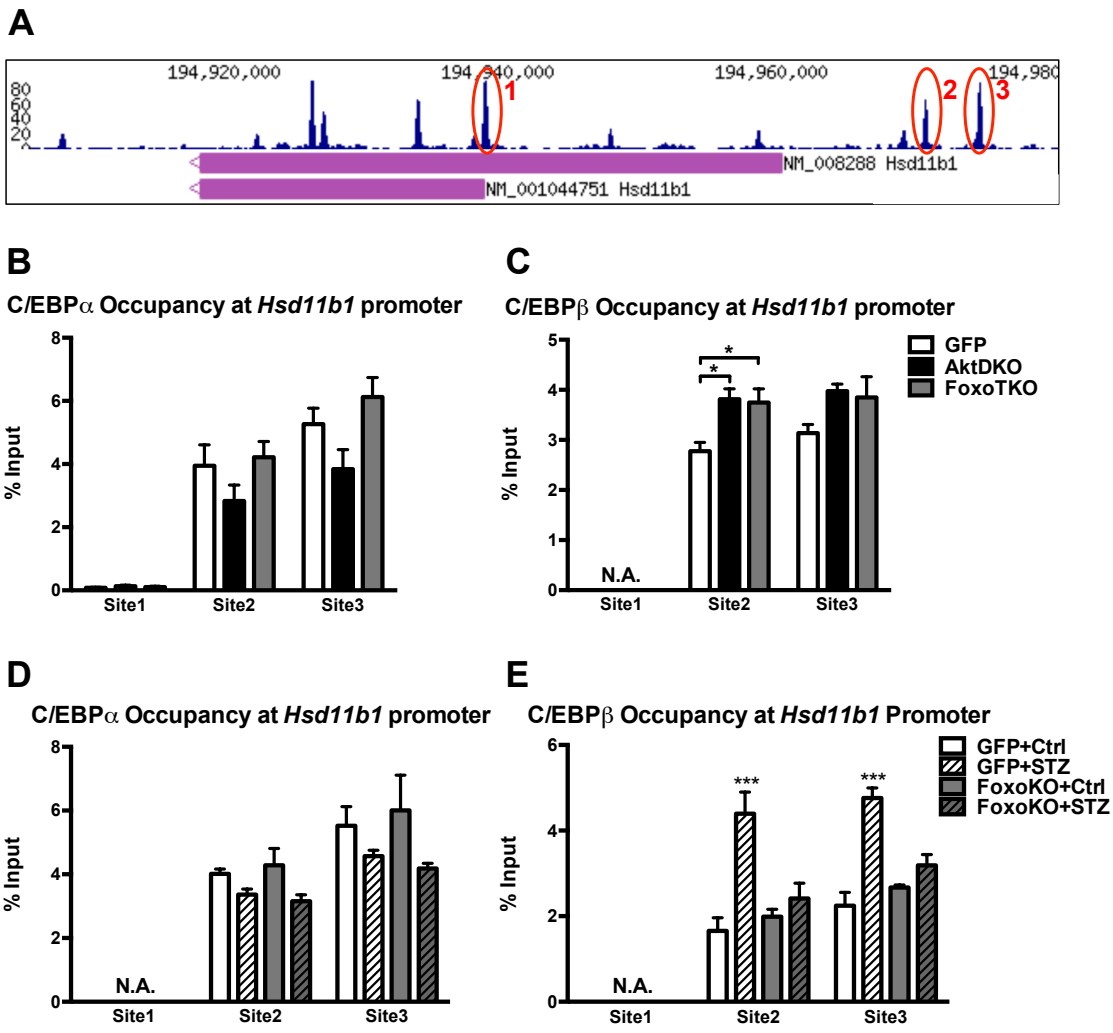

Figure 3.11: C/EBPα and C/EBPβ binding to the *Hsd11b1* promoter.

A. ChIP-seq profile for C/EBPβ at the *Hsd11b1* promoter. C/EBPβ binding sites selected for analyzing C/EBPα and C/EBPβ enrichment are indicated.

B,C. Hepatic enrichment of C/EBPα (B) and C/EBPβ (C) at the indicated sites of the *Hsd11b1* promoter in GFP control (GFP), liver-specific *Akt1*/*Akt2* double-knockout (AktDKO), and liver-specific *Akt1*/*Akt2*/*Foxo1* triple-knockout

(FoxoTKO) animals. n = 3; N.A., not available, and *p<0.05 vs. GFP by two-way ANOVA using Sidak post-test.

D,E. Hepatic enrichment of C/EBPα (D) and C/EBPβ (E) at indicated sites of the *Hsd11b1* promoter in GFP and liver-specific *Foxo1* knockout (FoxoKO) animals 9 days post an intra-peritoneal injection of either buffer (Ctrl) or streptozotocin (STZ) at 200mg per kg body weight. n = 3; N.A., not available, and ***p<0.001 vs. GFP+Ctrl by two-way ANOVA using Sidak post-test.

All values are expressed as mean ± SEM.

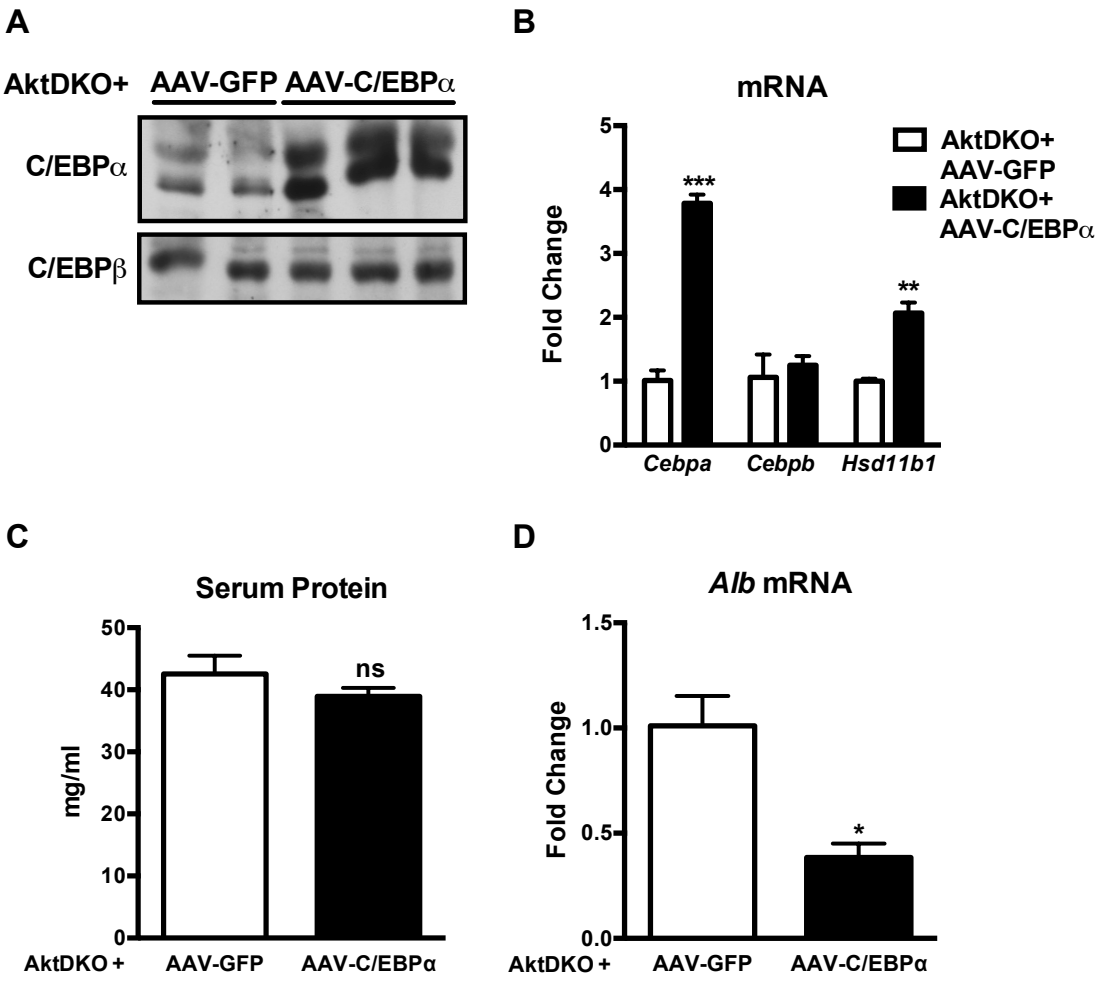

Figure 3.12: Overexpression of *Cebpa* in liver-specific *Akt1*/*Akt2* double-knockout (AktDKO) animals fails to rescue the reduced albumin production.

A. Western blots for C/EBPα and C/EBPβ in liver homogenates of AktDKO animals that either do (AAV-C/EBPα) or do not (AAV-GFP) overexpress *Cebpa*.

B-D. Hepatic gene expression of *Cebpa*, *Cebpb*, and *Hsd11b1* (B), serum protein concentration (C), and hepatic albumin mRNA level (D) in AktDKO animals that either do or do not overexpress *Cebpa*.

All values are expressed as mean ± SEM. n = 2-3; ns, not significant, *p<0.05 vs. AktDKO+AAV-GFP, **p<0.01 vs. AktDKO+AAV-GFP, and ***p<0.001 vs. AktDKO+AAV-GFP by two-tailed Student's t-test.

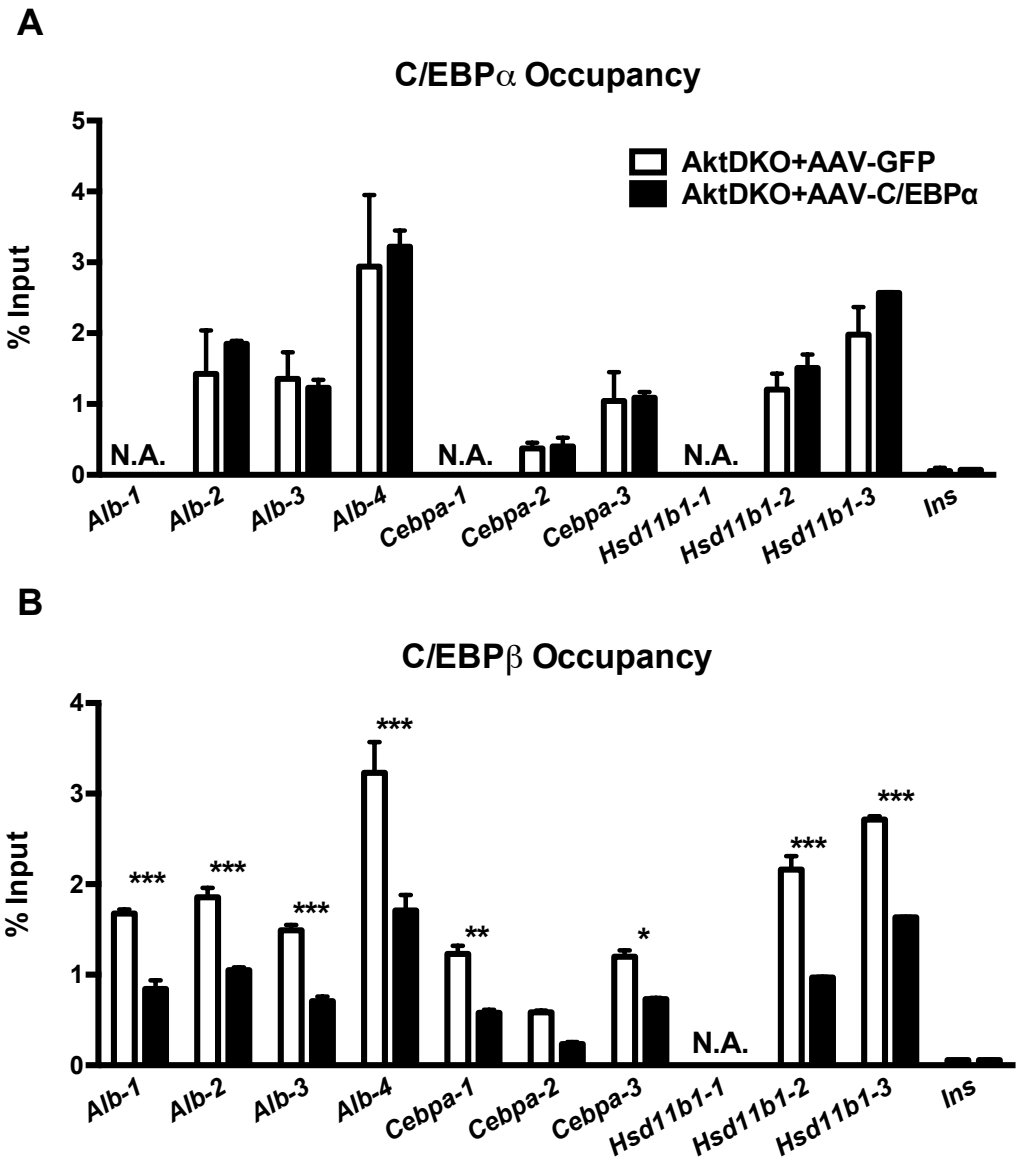

Figure 3.13: Overexpression of *Cebpa* in liver-specific *Akt1*/*Akt2* double-knockout (AktDKO) animals fails to rescue the reduced C/EBPα binding to the albumin promoter and further decreases C/EBPβ occupancy.

Hepatic enrichment of C/EBPα (A) and C/EBPβ (B) at indicated sites of the *Alb*, *Cebpa*, and *Hsd11b1* promoters (See Figure 3.9A, Figure 3.11A, and Figure

3.12A) in AktDKO animals that either do (AktDKO+AAV-C/EBPα) or do not (AktDKO+AAV-GFP) overexpress *Cebpa*. Ins serves as a negative control site not bound by C/EBPα.

All values are expressed as mean ± SD. n = 2; N.A., not available, *p<0.05 vs. AktDKO+AAV-GFP, **p<0.01 vs. AktDKO+AAV-GFP

Chapter 4

Summary and speculations

Insulin stimulates albumin transcription by inhibiting Foxo1

The effects of insulin on carbohydrate and lipid metabolism have been extensively characterized, but how insulin regulates protein metabolism remains largely unknown (Kimball et al., 1994; Tessari et al., 2011). Although it has been almost 30 years since it was first demonstrated that insulin controls serum albumin production in liver by stimulating albumin transcription (Lloyd et al., 1987), the detailed pathway has not been elucidated until the present study. Using liver-specific knockout models, we found that insulin signals directly on the liver through Akt to phosphorylate and inhibit Foxo1, which functions as a transcription repressor of the albumin gene. We also found that chronic activation of hepatic Foxo1 in Type 1 diabetic subjects is causal for the hypoalbuminemia phenotype in these individuals.

We speculate that this regulatory mechanism of insulin was evolved to limit nonessential biosynthesis during metabolic stress. Albumin mRNA and protein have a long half-life and therefore do not change with normal fasting and feeding, but decrease during diabetes and prolonged starvation. Under these conditions, nutrients for energy generation to sustain survival become extremely limited. Since that albumin is synthesized at a high rate (12-25 grams per day in a young healthy adult) (Fanali et al., 2012), it is essential to efficiently turn off its production to preserve the limited amino acids during an extended fast, and the most effective way to achieve this would be to reduce the level of mRNA message.

Foxo1 is a transcriptional repressor of the *Alb* gene

There still remains considerable uncertainty about the mechanism by which Foxo1 functions as a repressor of the albumin gene. Previous works have shown that the DNA-binding domain of Foxo is not required for Foxo to mediate gene repression, suggesting an indirect mechanism (Murphy et al., 2003; Schuster et al., 2010; Tepper et al., 2013). A recent study in C. *elegans* described PQM-1 as a transcription activator that exhibits reciprocal cellular localization as Foxo1, thereby presenting a possible mechanism in which Foxo1 activation leads to the nuclear exclusion of PQM-1 and subsequent gene downregulation (Tepper et al., 2013). However, it is not clear whether an orthologous mechanism exists in mammalian systems. Here, we propose three indirect mechanisms by which Foxo1 represses albumin gene expression: 1) by modulating the activity of another transcription factor via direct protein-protein interaction; 2) by inducing the expression of a transcriptional repressor; and 3) by mediating a metabolic change in the cell.

Foxo1 as a repressor via direct protein-protein interaction

Previous studies have extensively characterized the transcriptional regulation of *Alb* and identified C/EBPα, C/EBPβ, Hnf-1α, and Dbp as several liver-enriched transcription factors that stimulate albumin gene expression (Lichtsteiner et al., 1987; Maire et al., 1989). Our results show that Foxo1 activity is inversely correlated with the occupancy level of C/EBPα at the albumin

promoter. Although we did not perform co-immunoprecipitation experiments to directly demonstrate that Foxo1 and C/EBPα physically interact with each other, previous experimental data from other studies in adipocytes and neonatal liver suggests that such interaction is very likely (Qiao and Shao, 2006; Sekine et al., 2007). Given these data, we speculate that in the absence of insulin signaling, Foxo1 is nuclear and directly binds to C/EBPα; the interaction between C/EBPα and Foxo1 decreases C/EBPα binding to the albumin promoter, and thus downregulates albumin gene expression. This model is further supported by other examples in literature where Foxo1 directly interacts with transcription factors to inhibit their DNA binding (Christian, 2002; Deng et al., 2012; Dowell, 2003; Fan et al., 2009; Hirota et al., 2008; Ramaswamy et al., 2002; Van der Vos and Coffer, 2008). Taken together, this could indeed be the mechanism by which Foxo1 acts as a repressor for albumin transcription. It is important to note, however, that in the present study, we only examined how Foxo1 influences the DNA binding of C/EBPs. It is entirely possible that in addition to these transcription factors, Foxo1 also binds to and interferes with other transcription activators that regulate albumin gene expression.

Foxo1 as a repressor by inducing an intermediate repressor

In addition to regulating the transcription of insulin-responsive genes such as gluconeogenic enzymes, Foxo1 also regulates the expression of other transcription factors. Thus, it is possible that Foxo1 represses gene expression by inducing the expression of an intermediate repressor. Although no repressor

for albumin gene expression has been described to date, we found that Shp and Id3, repressors induced by Foxo1, may play a role in albumin gene regulation (Nakayama et al., 2006; Shin et al., 2012). We tested whether Shp and Id3 could mediate albumin gene repression downstream of Foxo1 in our models and found contradictory results. Specifically, while Shp and Id3 were induced in Akt-deficient livers in a Foxo1-dependent manner, this pattern was not observed in IR-deficient livers. This inconsistency suggests that regulation of Shp and Id3 is more complex and might involve factors other than Foxo1. Since albumin gene expression was decreased in both Akt-deficient and IR-deficient livers, it is unlikely that induction of Shp and Id3 was the mediator for albumin gene repression downstream of Foxo1. Additional studies might be necessary to determine whether other transcription repressors act downstream of Foxo1 to repress albumin expression.

Foxo1 as a repressor by mediating metabolic changes

Albumin expression in STZ-induced Type 1 diabetic mice was also decreased, and liver-specific deletion of *Foxo1* was sufficient to restore albumin expression to control levels in these mice. However, the mechanism by which Foxo1 represses albumin gene expression in this model was not immediately clear. Despite of the high Foxo1 activity, C/EBPα binding to the *Alb* promoter was unaffected, indicating that the reduction in albumin expression was not caused by decreased C/EBPα activity at the promoter. The discrepancy between this model and the liver-specific knockout models could be due to genetic strain

variation: we used mice of mixed background for the STZ studies, and our liver-specific knockout model mice were of pure *Bl6* background. In addition, the discrepancy is most likely caused by different pathogenesis of diabetes in these two models. STZ injection leads to β-cell death and therefore completely obliterates whole-body insulin signaling. In the liver-specific knockout models, on the other hand, disruption of insulin signaling originates specifically in the liver, and insulin resistance is subsequently developed in peripheral tissues. STZ-induced Type 1 diabetes represents a more severe and systemic disruption of metabolic homeostasis, thus it is challenging to determine the liver-specific mechanism of how Foxo1 represses albumin expression using this model.

Regardless, there must be a mechanism that explains our observation that albumin expression was inversely correlated with Foxo1 activity in STZ-induced Type 1 diabetic liver. One possibility is that the normalization of albumin transcription is downstream of the metabolic effects of inhibiting Foxo1. Numerous studies have shown that antagonizing or reducing hepatic Foxo1 in insulin resistant mice can significantly improve glucose tolerance and insulin action (Altomonte et al., 2003; Dong et al., 2008; Lu et al., 2012; Matsumoto, 2006; Matsumoto et al., 2007; Samuel et al.). For instance, earlier work from our laboratory shows that concomitant liver-specific deletion of Foxo1 normalizes the metabolic defects observed in mice with Akt-deficient liver (Lu et al., 2012). We speculate that defective albumin gene expression in STZ-induced diabetic liver may be normalized as a result of the improved metabolic homeostasis.

Foxo1 inhibition normalizes glucose utilization in the liver, possibly by increasing the expression of glucose kinase (Gck) and/or decreasing the expression of pyruvate dehydrogenase kinase 4 (Pdk4) (O-Sullivan et al., 2015). Gck catalyzes the first step of the glycolytic pathway, where dietary glucose becomes phosphorylated and retained inside the hepatocytes. Pdk4 phosphorylates and inhibits pyruvate dehydrogenase (Pdh), which converts pyruvate to acetyl-CoA, a key enzymatic step to shuttle carbohydrate into the citric acid cycle for lipid synthesis or energy generation. Therefore, inhibition of Foxo1 would lead to increased flux through the glycolytic pathway and the citric acid cycle, generating more energy and/or acetyl-coA in the cell. Increased energy charge in liver would alter activities of energy-sensing pathways such as AMP-activated protein kinase (AMPK) and cAMP responsive element binding protein (CREB), leading to transcriptional changes. In addition to energy generation and lipid synthesis, acetyl-CoA is also used to modify lysine residues of proteins, including transcription factors and histones. Acetylation of transcription factors has been shown to regulate their activity (Park et al., 2015). Histone acetylation is sensitive to cellular metabolism and plays an important role in gene regulation as well (Grunstein, 1997; Wellen et al., 2009). It is thus conceivable that either increased ATP or higher level of acetyl-CoA in hepatocytes could induce albumin gene expression.

Hepatic autophagy contributes to serum albumin production

Autophagy is an important quality control process that degrades and recycles damaged cellular proteins and organelles. In addition to its housekeeping role, autophagy also maintains metabolic homeostasis in various tissues and serves as a significant source of biosynthetic substrates and energy during metabolic stress (Kim and Lee, 2014; Rabinowitz and White, 2010; Yamada and Singh, 2012). In liver, insulin signaling turns off autophagy by activating mTORC1, which inhibits autophagy (Kim and Lee, 2014). Autophagy also modulates insulin action: suppressing autophagy impairs insulin signaling and inducing hepatic autophagy can increase insulin sensitivity in the liver (Yang et al., 2010). To our surprise, we found that hepatic autophagy, a catabolic process, positively contributes to serum albumin biosynthesis and secretion. Disrupting hepatic autophagy leads to a decrease in serum albumin level in overnight-fasted animals. In addition, autophagy can maintain some level of albumin production in livers with impaired Akt and mTORC1 activity.

Contribution of autophagy on serum albumin production occurs at a post transcription site. We speculate that autophagy supports albumin protein translation by supplying amino acids and/or energy generated from autophagic degradation. In addition, recent data suggests that co-localization of autophagosomes and mTORC1 facilitates the synthesis and secretion of proteins (Narita et al., 2011). Based on this observation, it is possible that hepatic autophagy also contributes to serum albumin production by augmenting its secretion. A pulse-chase experiment would be helpful to directly address whether autophagy contributes to albumin secretion. Incorporation of puromycin,

an aminoacylated-tRNA analog, into nascent peptide chain is a method alternative to radioactive amino acid labeling to study protein synthesis (Schmidt et al., 2009). Present at very low concentration (10μg/ml), puromycin does not inhibit protein synthesis and its incorporation is conveniently detected by immunoblotting. To measure albumin secretion in AktDKO and Atg5TKO hepatocytes, I will incubate the cells with puromycin for 10 minutes, followed by a 90-minute chase. At each time point within the chase period, albumin in the culture media will be immunoprecipiated, and levels of puromycin incorporation into albumin will be analyzed by western blot. If the appearance rate of puromycin-containing albumin in the media is decreased in Atg5TKO hepatocytes, then it suggests autophagy augments the secretory capacity of hepatocytes to contribute to protein production.

Serum albumin production is intact in Type 2 diabetes

Using our liver-specific knockout models, we found that albumin transcription is significantly reduced when insulin signaling is disrupted in the liver. It is important to recognize that in these models, insulin signaling is completely absent and is therefore not fully representative of Type 2 diabetic livers, where insulin action is impaired. To assess how albumin transcription is affected in insulin resistant livers, we measured serum albumin level and hepatic *Alb* mRNA in leptin-deficient (*ob*/*ob*) mice, a common model for obesity and Type 2 diabetes. We found that unlike what we observed in liver-specific knockout models, serum albumin transcription and secretion are completely normal in

ob/*ob* mice. Consistent with our observation, an earlier study also showed that serum albumin production is normal in Type 2 diabetic patients (Tessari et al., 2006b). Taken together, we conclude that regulation of serum albumin production by insulin is intact in Type 2 diabetes despite of the hepatic insulin resistance.

Type 2 diabetic patients exhibit the “classic triad” of hyperinsunlinemia, hyperglycemia, and hypertriglyceridemia. However, this triad of metabolic defects breaks down when insulin signaling is disrupted at the insulin receptor level (Michael et al., 2000). This apparent paradox is explained by the dual action of insulin in the liver: insulin suppresses glucose production by phosphorylating and inhibition Foxo1, which induce the expression of key gluconeogenic enzymes; insulin also activates hepatic lipogenesis to increase lipid synthesis and secretion. To explain the simultaneous elevation of glucose and lipid production in liver in Type 2 diabetes, Brown and Goldstein suggested a model of “selective insulin resistance”, where insulin is unable to inhibit Foxo1 to suppress gluconeogenesis but continues to sustain lipogenesis (Brown and Goldstein, 2008). However, if this model is correct, Foxo1 should remain active in leptin-deficient mice, and should thus repress albumin expression. Since we found that albumin expression in these animals was normal, our data does not support the hepatic selective insulin resistance model.

In an alternative view, Otero and colleagues suggested that the primary phenotypic driver for Type 2 diabetes is altered substrate delivery to the liver and hepatic nutrient handling, and not impaired insulin action *per se*

(Otero et al., 2014). This model is supported by experimental evidence indicating that failure of insulin to suppress gluconeogenesis in liver is driven by substrate uptake and not gene expression (Catchpole et al., 2007; Edgerton et al., 2009; Ramnanan et al., 2010; Shawn C Burgess, 2007). In this model, insulin-mediated Foxo1 inhibition is likely intact in Type 2 diabetes, which is consistent with our observation that albumin expression is normal in *ob*/*ob* animals.

Chapter 5

Materials and Methods

Animals

All experiments were performed in male mice that were 10-12 weeks of age. The *Ir*$^{loxP/loxP}$, *Ir*$^{loxP/loxP}$; *Foxo1*$^{loxP/loxP}$, *Akt1*$^{loxP/loxP}$; *Akt2*$^{loxP/loxP}$, *Akt1*$^{loxP/loxP}$; *Akt2*$^{loxP/loxP}$; *Foxo1*$^{loxP/loxP}$, and *Foxo1*$^{loxP/loxP}$ mice have been described previously (Leavens and Birnbaum, 2011; Matsumoto, 2006; Michael et al., 2000). To generate liver-specific knockouts, an adeno-associated virus expressing either GFP or Cre recombinase driven by the promoter of liver-specific gene thyroxine binding globulin (TBG) was injected into the above mice at 8-10 weeks of age (1 x 10^{11} genomic copies per mouse). Experiments were performed 2 weeks post virus injection. For fasting-refeeding experiments, mice were deprived of food for 16 hours (4pm to 9am). The fasted group was sacrificed at 9am, and the refed group was fed *ad libitum* for 4 hours with normal chow (Laboratory Rodent Diet, Cat. 5001) before sacrifice. All animal experiments were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee in accordance with NIH guidelines.

Liver lysates/nuclear extract extraction and Western blotting

Post sacrifice, livers were dissected, freeze-clamped, and stored at -80°C. Whole cell lysates were prepared by homogenizing frozen liver samples in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.6, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease and phosphatase inhibitors). To detect Foxo1, liver nuclear extracts were prepared using the NE-

PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Cat. 78833). Cleared lysates and nuclear extracts were resolved by SDS-PAGE (10-12% acrylamide gel, constant voltage of 100V), transferred onto nitrocellulose membranes, probed with various antibodies (IR, Cell Signaling, Cat. 3025S; Foxo1, Cell Signaling, Cat. 9454S; Akt1, Cell Signaling, Cat. 2967; Akt2, Cell Signaling, Cat. 2964S; Actin, Abcam, Cat. ab6276) and visualized with either IRDye secondary antibodies (LI-COR Biosciences, Cat. 926-32213 and 926-68022) or ECL Western blotting detection reagents (Thermo Scientific, Cat. 32106).

Primary hepatocytes isolation and in vitro albumin secretion assay

Primary hepatocytes were isolated as previously described (Miller et al., 2013). Cells were plated on collagen-treated plates in DMEM supplemented with 10% fetal bovine serum. After a 2-3 hr attachment period, cells were washed twice with PBS and incubated in serum-free Krebs-Ringer Bicarbonate Buffer (Sigma-Aldrich, Cat. K4002) supplemented with 20mM HEPES, pH 7.4 and 0.5% BSA for 2 hours. Media was collected and hemoglobin (Sigma Aldrich, Cat. H2625) was added as a carrier protein (final concentration of 0.1%, w/v). For trichloroacetic acid (TCA) precipitation, 1 volume of 100% TCA (w/v) was added to 4 volume of sample to precipitate total protein. The protein pellet was washed twice in ice-cold acetone, dried, and re-suspended in Laemmli sample buffer (volume adjusted based on cellular protein content). Albumin in the samples was

then measured by Western blotting (Anti-Alb, Nordic Immunology, Cat. RAM/Alb/7s).

mRNA isolation and real-time PCR

Total RNA was isolated from frozen livers or primary hepatocytes using the Nucleospin RNA Mini Kit (Clontech Labs, Cat. 740955.250). cDNA was synthesized using M-MLV reverse transcriptase (New England Biolabs, Cat. M0253S). Liver cDNA from transgenic mice expressing a constitutively active Foxo1 was a generous gift from Dr. Terry G. Unterman (University of Illinois at Chicago College of Medicine) (Deng et al., 2012). The relative expression of genes of interest was quantified by real-time PCR using the SYBR Green Dye-based assay.

Serum albumin measurement

Blood samples were collected post sacrifice by cardiac puncture. After allowing the blood to clot, the samples were centrifuged to separate the sera. Albumin levels were measured using the BCG Albumin Assay Kit (Sigma Aldrich, Cat. MAK124).

Streptozotocin-induced Type 1 diabetes

At 8 to 10 weeks of age, *Foxo1*$^{loxP/loxP}$ mice received a retro-orbital injection of adeno-associated virus encoding either GFP or Cre recombinase at 1

x 10^{11} genomic copies per mouse. 5 days after virus injection, mice received an intra-peritoneal injection of either control buffer (0.1 M citrate, pH 4.5) or streptozotocin (EMD Chemicals, Cat. 572201) at 200 mg per kg body weight. Mice were sacrificed 2 weeks after virus injection (9 days after STZ injection).

Chromatin immunoprecipitation (ChIP) assay

Liver chromatin was prepared as previously described (Tuteja et al., 2008). Immunoprecipitations were performed using anti-C/EBPα (Santa Cruz, Cat. sc-61, 10μg per IP). Real-time PCR oligos used to measure occupancy are listed below.

Statistical analysis

All values are expressed as mean ± SEM. Two-way ANOVA with Bonferroni posttest was used if multiple conditions were involved when comparing different genotypes. Two-tailed, unpaired Student's t-test was used when only two groups of data were concerned.

Real-time PCR oligos used to measure C/EBPα occupancy

Alb-1f	CGCAAGGGATTTAGTCAAACAAC
Alb-1r	AACCATACTTACCTCGCATTTCA
Alb-2f	TCCCAGACCCATCAATTGTG
Alb-2r	TCCTGGCTCTTAGATTGCTCA
Alb-3f	AGCTAACCTTCTGTCCTAGTGG
Alb-3r	TGAACTCTGACTCACGATGGA
Alb-4f	ACAGAGGGTTGGATGGACAC
Alb-4r	CCTCATTACCTTTGTGCACCA
Cebpa-1f	AGGAGTCAGTGGGCGTTG
Cebpa-1r	GTCTTAGAGCCCGCCTTCTC
Cebpa-2f	TCCGTCTTCCTATACCAGTCTG
Cebpa-2r	CACCCAGTCCCAGTGATAGT
Cebpa-3f	CTTCAAGCAGATCCCAGGAAC
Cebpa-3r	CTAGTTCCGACCCTTCCTCC
Hsd11b1-1f	ATGGGCATCCTACAATTTCCT
Hsd11b1-1r	GGTCAGAGAACATTGGGGAAC
Hsd11b1-2f	CCAACTGGCCAGAAAATTGC
Hsd11b1-2r	ATTGGCACCCTTTCCCCTAA
Hsd11b1-3f	GGCTCGTCCTTGGCTTAGA
Hsd11b1-3r	GCTGGTGGTGGAAGTGAAAG

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